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Acetylsalicylic acid potentiates the antinociceptive effect of morphine in the rat: involvement of the central serotonergic system

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Abstract

Acetylsalicylic acid and morphine are the most widely distributed and most frequently used drugs in the relief of pain, but their analgesic activity has adverse side-effects. Mixtures containing these two drugs are frequently used to relieve mild to moderate pain despite the paucity of relevant experimental evidence so far published. We set out to study the possible antinociceptive effect of a combination of subactive doses of the two drugs in rats. A combination of low doses of acetylsalicylic acid (50 mg/kg i.p.) and morphine (3 mg/kg s.c.) was administered and the pain threshold was evaluated in the hot-plate and formalin tests, and 5-HT_2 receptor binding capacity, 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured in the cortex and pontine areas of the brain. The combination of acetylsalicylic acid and morphine had an analgesic effect in both tests that was associated with an increase in 5-HT levels and a decrease in 5-HT_2 receptors in the cortex. These effects were either completely abolished or partially prevented by i.p. pretreatment with naloxone (1 mg/kg i.p.). Our results demonstrate that subactive doses of acetylsalicylic acid and morphine can exert analgesic and biochemical effects when given in combination in the rat and suggest an involvement of serotonergic and opiatergic systems.

Keywords: Acetylsalicylic acid; Morphine; Antinociception; 5-HT (5-hydroxytryptamine, serotonin); 5-HIAA (5-hydroxyindoleacetic acid); 5-HT_2 receptor; Brain

1. Introduction

Mixtures containing two analgesic agents are used in therapeutics in an attempt to produce greater analgesic effects. However, little research has been undertaken to determine the effectiveness and advantages of such combinations in clinical trials (Lopez-Munoz et al., 1994), and conflicting results have been reported in experimental studies. These differences may be due to different dose combinations, to the use of a variety of drugs belonging to different chemical classes and to the different models used to evaluate the analgesic effect (Godefroy et al., 1986).

We recently observed that acetylsalicylic acid displays antinociceptive activity both in the hot-plate test and in the formalin test in the rat, while significantly increasing the brain serotonin content and reducing the number of 5-HT_2 receptors in cortical membranes (Sandrini et al., 1995). The depletion of central 5-hydroxytryptamine (5-HT) levels induced by parachlorophenylalanine significantly reduces this analgesic effect and abolishes the changes in serotonin concentration and in the number of 5-HT_2 receptors, thus suggesting the importance of the serotonergic system in the antinociceptive activity of acetylsalicylic acid (Pini et al., 1995).

Serotonin is known to play an important role in the central and peripheral mechanisms of nociception (Warner et al., 1990); moreover, 5-HT facilitates or has no effect on reducing analgesia depending upon the experimental conditions (Murphy et al., 1992). In the central nervous system the serotonergic pathways that modulate nociception might be involved in the mechanism of the antinociceptive action of opioids (Basbaum and Fields, 1985). There is evidence that the serotonergic system can play a role in the antinociceptive mechanism of some non-steroidal anti-inflammatory drugs (NSAIDs), whereas it has been proposed that other neurotransmitter systems, including opioidergic pathways, may be involved in the

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central analgesic effect of this class of drugs (Björkman, 1995). Morphine stimulates 5-HT release via a supraspinal action (Bineau-Thourottes et al., 1984) and 5-HT depletion in the central nervous system (CNS) decreases the analgesic effect of morphine (Bodnar et al., 1981). Thus, it can be assumed that morphine exerts its analgesic effect, at least in part, through the serotonergic system (Drissen and Reiman, 1992; Yang et al., 1994). Since morphine and acetylsalicylic acid may have common pathways in their mechanism of action, we set out to discover whether the combination of the two drugs, at subactive doses, results in a potentiation of analgesic efficacy.

The aim of this work was to assess: (1) the analgesic effect of combinations of acetylsalicylic acid and morphine, at subactive doses, in the hot-plate and formalin tests; (2) the changes in serotonin and 5-hydroxyindoleacetic acid (5-HIAA) levels and 5-HT_2 receptors in rat brain membranes; (3) the influence of pretreatment with naloxone on the behavioral and biochemical changes induced by the combination of the two drugs.

2. Materials and methods

2.1. Animals

Adult male rats (Harlan-Nossan, SPF, Correzzano, Italy), weighing 180–200 g at the beginning of the experiments, were housed in plexiglass cages, four per cage, with free access to food and water, and maintained on a 12-h dark/light cycle (light on at 0700) under controlled environmental conditions (temperature, 22 ± 1°C; humidity, 60%). The ethical guidelines for the 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. 27/01/1982, No. 116).

2.2. Drug treatment

The rats were randomly divided into groups of eight animals each. Naloxone (1 mg/kg in 2 ml of sterile saline) or saline was injected i.p.; acetylsalicylic acid (50 or 100 mg/kg i.p.), morphine 1, 2, 3 and 5 mg/kg s.c. dissolved in saline or saline was injected 10 min after pretreatment with naloxone. Rats were injected with morphine 10 min after acetylsalicylic acid and subjected to the algesimetric tests 20 min later. Five more groups were tested in the hot-plate test by using the doses of 50, 100, 200, 300 and 400 mg/kg of acetylsalicylic acid to obtain a dose–response curve. At the end of the experiments the rats were anesthetized and decapitated, and their blood and brains were removed and stored until required for analysis. Two additional groups of rats were tested for motor activity after treatment with either morphine 3 plus acetylsalicylic acid 50 mg/kg or saline.

2.3. Hot-plate test

The hot-plate consisted of an electrically heated surface (Socrel DS-35, Ugo Basile, Comerio, VA, Italy) kept at a constant temperature of 54 ± 0.8°C. The latencies for paw licking or jumping were recorded for each animal. The baseline latency in the hot-plate test ranged from 5.9 ± 0.8 to 6.3 ± 0.9 s (analysis of variance test (ANOVA), $P > 0.5$). The analgesic efficacy of the drugs was evaluated as %MPE (maximum possible effect) according to the formula: $(TL - BL)/(45 - BL) \times 100$, where TL = test latency; BL = baseline latency; 45 = cutoff time in seconds.

Immediately after the last pain threshold measurement, the animals were anesthetized with ethyl ether and decapitated; the blood was collected and the serum was stored at -20°C.

The brains were removed, weighed and stored at -80°C until required for assay.

2.4. Formalin test

Two hours before testing, the animals were placed individually in standard cages and, after the adaptation period, 50 μl of 5% formalin solution was injected subcutaneously into the dorsal surface of the left hindpaw by means of a microsyringe with a 26-gauge needle. Pain behavior was monitored for a period of 40 min, the number of flinches/shakes of the injected paw being summed at 5-min intervals starting at time 0. Two phases of spontaneous flinching behavior were observed: phase 1 began immediately after formalin injection and lasted for 10 min and phase 2 began at 11 min and ended 40 min after formalin injection.

To avoid the possible interference of room temperature on skin temperature, all experiments were performed at a room temperature of 22 ± 1°C (Hole and Tjølsen, 1993).

2.5. Motor activity

The experiments were performed between 0900 and 1200 in a soundproof room by experienced observers unaware of the treatments. Motor activity was measured in an activity cage by means of an ultrasound apparatus (Cibertec, Barcellona, Spain) placed on the lid of the cage. The number of movements was recorded continuously for 1 h after an adaptation period of 30 min.

2.6. Salicylates assay

Salicylates were determined in the sera by fluorescence polarization immunoassay (FPIA). A TDx analyzer was used for drug evaluation (Abbot Laboratories, Chicago, IL, USA).

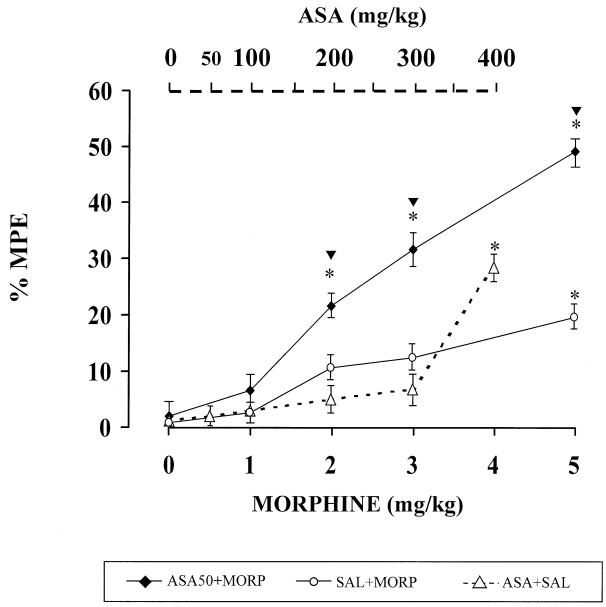


Fig. 1. Antinociceptive effect of acetylsalicylic acid (ASA) and morphine (MORP) in combination in the hot-plate test. %MPE represents the percentage of the maximum possible effect. MORP (1–5 mg/kg s.c.) was administered 10 min after ASA (50 mg/kg i.p.) and the rats were tested 20 min after morphine or 30 min after ASA alone. Values are means of 10 rats for each group. $* P < 0.05$ vs. control (ANOVA followed by Student–Newman–Keuls' test). $^{\blacktriangledown} P < 0.05$ vs. MORP alone. (ANOVA followed by Student–Newman–Keuls' test).

2.7. Serotonin determination

5-HT and 5-HIAA concentrations were measured by reverse-phase high-performance liquid chromatography (HPLC), according to Grossi et al. (1990) with modifications.

The thawed brain areas were homogenized with an ultrasonic cell disrupter in 0.1 M $HClO_4$ containing 4 mM $NaHSO_4$ (10 μl/mg of wet weight) and centrifuged at $2000 \times g$ for 15 min at 4°C. After centrifugation, the acid supernatant was filtered through 0.22-μm filters before analysis.

5-HT and 5-HIAA assays were performed with a Beckman System Gold high-performance liquid chromatograph (Beckman Instruments, San Ramon, CA, USA) equipped with a ESA Coulochem II Multi-Electrode high sensitivity electrochemical detector (ESA, Bedford, MA, USA) with conditioning cell set at −0.75 V, detector 1 set at +0.05 V and detector 2 set at +0.25 V, response 2, gain 10×5.

A reverse-phase C-18 10 cm × 4.6 mm Hypersil column (Labservice Analytical, Bologna, Italy) packed with 3 μm ODS was used. The mobile phase, consisting of methanol 15%, acetonitrile 8% and 50 mM NaH_2PO_4, pH 2.8, with 0.2 mM ethylenediaminetetracetic acid disodium salt and 200 mg/l sodium octyl sulphate, was pumped at a rate of 1 ml/min. 3,4-Dihydroxybenzylamine, the internal standard, 5-HT and 5-HIAA were used as standards. Standards and samples were quantitated according to the analyte/3,4-dihydroxybenzylamine ratio in a calibration curve.

2.8. Binding assay

The characteristics of 5-HT_{1A} binding sites were evaluated according to Gulati and Bhargava (1990) using six concentrations of [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin, [^{3}H]8-OH-DPAT, (0.18–6 nM) (specific activity 142.9 Ci/mmol).

The characteristics of 5-HT_2 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 $1300 \times g$ for 10 min at 4°C. This procedure was repeated, the combined sucrose supernatants were diluted with 10 ml of 50 mM Tris–HCl, pH 7.7, and the suspension was centrifuged at $3500 \times g$ for 10 min. The pellet was resuspended in 20 ml of Tris–HCl buffer and centrifuged once at $50\,000 \times g$ 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 [^{3}H]ketanserin (six increasing concentrations in 10% ethanol), methysergide (10 μM, dissolved in Tris–HCl buffer to define non-specific binding) or Tris buffer at 37°C for 15 min. The mixture was filtered under reduced pressure through Whatman GF/B glass fiber filters, which had been soaked for 5 min in 0.5% polyethylenimine, by using a Millipore vacuum pump and rapidly rinsed twice with 5 ml ice-cold Tris buffer. The filters were transferred

Table 1
Influence of naloxone (NAL) treatment on the antinociceptive effect of acetylsalicylic acid (ASA) and morphine (MORP) in the hot-plate test

	Treatment					
	SAL + SAL	NAL + SAL	ASA + MORP3	ASA + MORP5	NAL + ASA + MORP3	NAL + ASA + MORP5
%MPE	1.36 ± 3.4	1.12 ± 2.1	32.90 ± 5.7[a]	47.90 ± 5.7[b]	6.70 ± 2.7	7.45 ± 2.3

NAL (1 mg/kg i.p.) or SAL (2 ml/kg) was injected 10 min before ASA (50 mg/kg i.p.) plus MORP (3 or 5 mg/kg s.c.) treatment.
Hot-plate test started 20 min after the last treatment.
%MPE = percentage of the maximum possible effect.
ANOVA followed by Student–Newman–Keuls' test.
[a] $P < 0.05$ vs. SAL.
[b] $P < 0.05$ vs. MORP5.

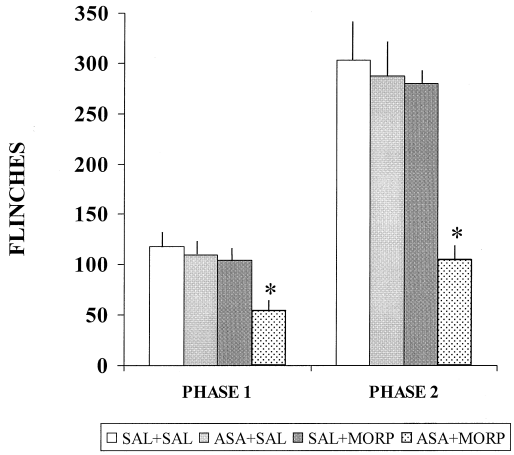


Fig. 2. Antinociceptive effect of a combination of acetylsalicylic acid (ASA) and morphine (MORP) in the formalin test. Saline (SAL, 2 ml/kg), ASA (50 mg/kg i.p.) and MORP (3 mg/kg s.c.) were administered 10 min after each other and the rats were tested 20 min after morphine. Each histogram represents the total number of flinches (mean ± S.E.M.) in phase 1 and in phase 2. $* P < 0.05$ vs. control values (ANOVA followed by Student–Newman–Keuls' test).

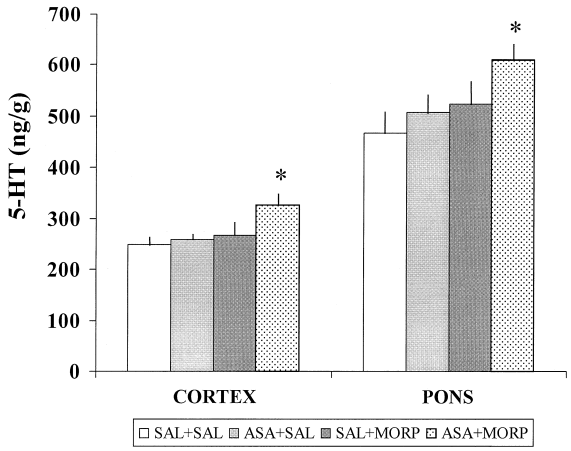


Fig. 4. The effect of the combination of acetylsalicylic acid (ASA) and morphine (MORP) on serotonin content in cortical and pontine membranes. Saline (SAL, 2 ml/kg), ASA (50 mg/kg i.p.) and MORP (3 mg/kg s.c.) were administered 10 min after each other. The rats were killed at the end of the hot-plate test and brain areas were weighed and frozen at −80°C until assayed. Values are expressed as means ± S.E.M. for six rats for each group. $* P < 0.05$ vs. control values (ANOVA followed by Student–Newman–Keuls' test).

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 [^{3}H]ketanserin (specific activity, 87.5 Ci/mmol). The specific binding was 60 to 70% of the total binding for [^{3}H]ketanserin.

Competition experiments were performed using 10 concentrations between 0.1 nM to 10 μM of unlabelled acetylsalicylic acid to displace binding of 1 nM [^{3}H]naloxone (specific activity 58.2 Ci/mmol) according to the method of Windh et al. (1995).

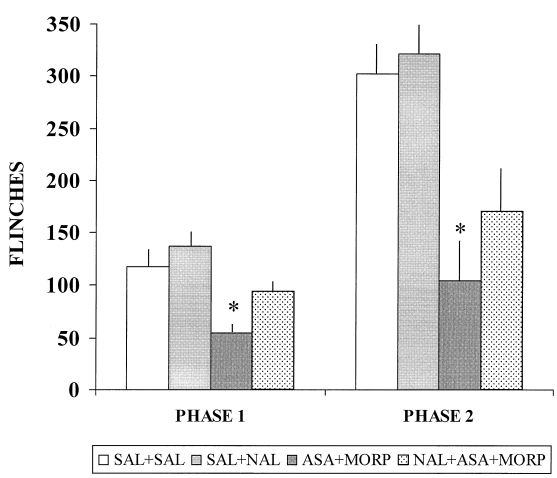
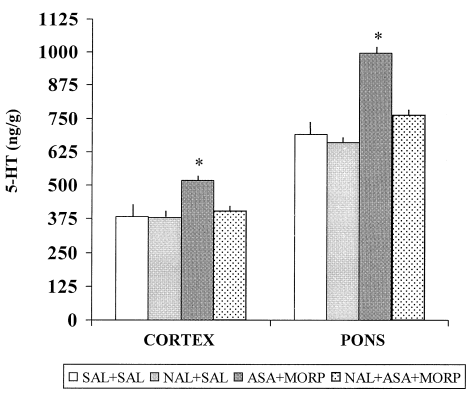


Fig. 3. Influence of naloxone (NAL) pretreatment on the antinociceptive effect of a combination of acetylsalicylic acid (ASA) and morphine (MORP) in the formalin test. NAL (1 mg/kg i.p.) or saline (SAL, 2 ml/kg) was injected 10 min before ASA (50 mg/kg i.p.) plus MORP (3 or 5 mg/kg s.c.) treatment. Each histogram represents the total number of flinches (mean ± S.E.M.) in phase 1 and in phase 2. $* P < 0.05$ vs. control values (ANOVA followed by Student–Newman–Keuls' test).



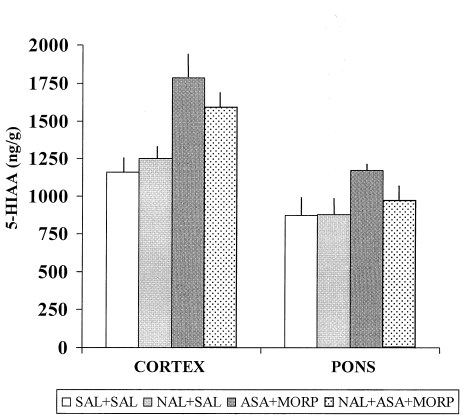


Fig. 5. Influence of naloxone (NAL) pretreatment on the effect of acetylsalicylic acid (ASA) and morphine (MORP) on serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) levels in the rat brain. NAL (1 mg/kg i.p.) was administered 10 min before ASA and 20 min before MORP; the rats were killed at the end of the hot-plate test and brain areas were weighed and frozen at −80°C until assayed. Each value represents the mean ± S.E.M. of six separate experiments. $* P < 0.05$ vs. SAL (ANOVA followed by Student–Newman–Keuls' test).

2.9. Statistics

The results obtained for motor activity were analyzed with Student's *t*-test. The results of binding experiments were analyzed according to the method of Rosenthal (1967). The equilibrium dissociation constant (k_D) and maximum number of binding sites (B_{max}) were evaluated individually for each sample with six concentrations of labelled drug. A two-way analysis of variance followed by 2×2 factorial analysis by means of orthogonal comparisons was used to analyze the effect of acetylsalicylic acid + morphine treatment, naloxone treatment and their combination (Snedecor and Cochran, 1980).

The data were examined by ANOVA, followed by Student–Newman–Keuls' test when the effects of naloxone and acetylsalicylic acid + morphine were being evaluated separately.

2.10. Drugs

Acetylsalicylic acid as lysine salt was purchased from Sanofi-Winthrop, Milano, Italy, while morphine hydrochloride, 5-HT, 5-HIAA, 3,4-dihydroxybenzylamine and naloxone were purchased from Sigma, St. Louis, MO, USA.

[^{3}H]8-hydroxy-2-(di-*n*-propyl-amino)tetralin ([^{3}H]8-OH-DPAT), [^{3}H]ketanserin and [^{3}H] naloxone were from Du Pont NEN, Milan, Italy. Formalin was obtained through Bracco Chemical, Milan, Italy.

3. Results

Fig. 1 shows the effect of increasing doses of acetylsalicylic acid (50–400 mg/kg i.p.) and the effect of a subactive dose of acetylsalicylic acid (50 mg/kg i.p.) administered together with increasing doses of morphine in the hot-plate test. The %MPE increased significantly only at the dose of 400 mg/kg of acetylsalicylic acid or 5 mg/kg s.c. of morphine, while the combination of the two drugs provoked a significant increase in %MPE at the doses of 2, 3 and 5 mg/kg of morphine: only the effect of 3 and 5 mg/kg was potentiated by combination with acetylsalicylic acid ($F(1,32) = 5.87$; $P < 0.01$); ($F(1,32) = 7.01$; $P < 0.01$). Naloxone (1 mg/kg i.p.) significantly prevented the antinociceptive effect of acetylsalicylic acid plus morphine 3 or 5 mg/kg on the %MPE (Table 1); the interaction test showed a negative interaction ($F(1,28) = 13.9$; $P < 0.01$); ($F(1,28) = 27.4$; $P < 0.01$).

We chose the combination of acetylsalicylic acid 50 mg/kg and morphine 3 mg/kg to test antinociceptive activity in the formalin test, because these doses alone did not exert a significant analgesic effect and the combination seemed to be the most suitable to study a possible potentiation of the two drugs. This combination had a significant effect in the formalin test: the interaction test showed significant potentiation in the first phase ($F(1,24) = 5.94$; $P < 0.05$), but not in the second ($F(1,24) = 1.08$, ns), thus indicating that in this phase there was only a summation effect (Fig. 2). Naloxone prevented the effect of the combination in the first phase of the test ($F(1,24) = 6.07$, $P < 0.05$), but not in the second phase ($F(1,24) = 3.5$, ns) (Fig. 3). When the dose of acetylsalicylic acid was increased to 100 mg/kg, the combination with morphine 3 mg/kg provoked a significant potentiation even in the second phase of the formalin test ($F(1,24) = 8.76$; $P < 0.01$) (data not shown). None of these combinations affected motor activity, the number of movements being 1378 ± 60 for the combination acetylsalicylic acid 50 mg/kg and morphine 3 mg/kg and 1324 ± 48 for the control group.

The levels of serotonin in the cortex and in the pons were increased by a combination of acetylsalicylic acid and morphine, while they did not change when the drugs

Table 2
Influence of naloxone (NAL) treatment on the effect of a combination of acetylsalicylic acid (ASA) and morphine (MORP) on the characteristics of 5-HT$_2$ receptors in the rat brain

Treatment	Cortex		Pons	
	B_{max} (fmol/mg protein)	K_d (nM)	B_{max} (fmol/mg protein)	K_d (nM)
SAL + SAL	230.0 ± 8.0	1.66 ± 0.27	42.4 ± 5.9	1.24 ± 0.10
NAL + SAL	267.4 ± 11.7	1.46 ± 0.10	n.d	n.d.
ASA + SAL	248.2 ± 9.4	1.55 ± 0.30	47.5 ± 6.4	1.46 ± 0.10
SAL + MORP	275.2 ± 14.3	1.44 ± 0.10	58.4 ± 7.4	1.51 ± 0.10
ASA + MORP	175.8 ± 9.9[a]	1.53 ± 0.23	38.4 ± 3.4	1.36 ± 0.10
NAL + ASA + MORP	202.8 ± 7.8	1.06 ± 0.13	n.d.	n.d.

Rat were injected with SAL (2 ml/kg), NAL (1 mg/kg i.p.), ASA 850 mg/kg i.p.) and MORP (3 mg/kg s.c.). They were killed at the end of the experiment and the brain were stored at −80°C until assayed. Each value represents the mean ± S.E.M. of six separate experiments and were derived form Rosenthal plot.

B_{max}, maximum binding capacity.

K_d, equilibrium dissociation constant.

ANOVA followed by Student–Newman–Keuls' test.

[a] $P < 0.05$ vs. SAL + SAL.

Table 3
Effect of a treatment with acetylsalicylic acid (ASA) and morphine (MORP) on the characteristics of 5-HT_{1A} receptors in the cortex of the rat brain

Treatment	B_{max} (fmol/mg protein)	K_d (nM)
SAL + SAL	269.3 ± 27.3	1.8 ± 0.3
ASA + SAL	264.1 ± 10.7	1.7 ± 0.3
SAL + MORP	212.7 ± 19.2	1.8 ± 0.3
ASA + MORP	266.8 ± 26.9	2.1 ± 0.3

Rat were injected with SAL (2 ml/kg), ASA (50 mg/kg i.p.) and MORP (3 mg/kg s.c.). They were killed at the end of the experiment and the brain were stored at −80°C until assayed. Each value represents the mean ± S.E.M. of six separate experiments and were derived from Rosenthal plot.
B_{max}, maximum binding capacity (fmol/kg protein).
K_d, equilibrium dissociation constant (nM).
ANOVA $P > 0.05$.

were given alone (Fig. 4). The interaction test showed a significant potentiation in the cortex ($F(1,20) = 5.71$; $P < 0.01$), but not in the pons ($F(1,20) = 1.3$, ns). The levels of 5-HIAA in the cerebral cortex and in the pons were increased but not significantly; this could be due to the large variability of the data obtained. Naloxone prevented the increase in serotonin levels in the cortex ($F(1,20) = 5.18$; $P < 0.05$) (Fig. 5).

The combination of the same doses of acetylsalicylic acid and morphine provoked a decrease in the number of 5-HT_2 receptors in the cortex ($F(1,20) = 7.0$; $P < 0.01$), but not in the pons, whereas neither drug changed the B_{max} in the two areas when given alone. Naloxone partially prevented the decrease in the B_{max}, but the interaction test showed no statistically significant effect ($F(1,20) = 0.93$, ns) (Table 2). The affinity constant remained unchanged. The characteristics of 5-HT_{1A} receptors were never affected either in the cortex or in the pons by either acetylsalicylic acid or morphine alone or in combination (Table 3). Acetylsalicylic acid was not able to compete for [^{3}H]naloxone binding sites. Competition experiments demonstrated that the maximum effect of displacement with respect to morphine was 7% for the cortex and 4% for the pons.

In order to avoid any pharmacodynamic interaction between naloxone, acetylsalicylic acid and morphine, we evaluated the levels of salicylates when the drugs were given in combination. The levels were 128.5 ± 7.5, 144.12.4 and 130.8 ± 4.3 μg/dl for acetylsalicylic acid 50 mg/kg, acetylsalicylic acid + morphine 3 mg/kg and naloxone + acetylsalicylic acid + morphine, respectively ($P = 0.80$, ns: Student's t-test).

4. Discussion

The evaluation of antinociceptive drug activity in animal models depends on several factors, such as the type of stimulus, species and strain of animals, and the type of recording device used in the experimental model (Abramson and Weissman, 1989).

Nevertheless, the hot-plate test, as used in our previous studies on the antinociceptive effect of phenazone (Sandrini et al., 1993) and paracetamol (Pini et al., 1996), is generally considered a reliable method of measuring the activity of non-steroidal anti-inflammatory drugs.

The behavioral response to the injection of formalin is biphasic, with an acute phase followed by a quiescent period and then a prolonged response with a maximum between 10 and 40 min (Malmberg and Yaksh, 1992). It has been suggested that the early phase is caused by the direct effect of formalin on nociceptors, whereas the second phase is a tonic response in which inflammatory processes are involved and neurons of the dorsal horn of the spinal cord are activated (Tjølsen et al., 1991). No obvious inflammatory state was found 10 min after formalin injection. Thus, the antinociceptive non-antiinflammatory properties of drugs can be evaluated immediately after injection of formalin in the subsequent 10 min (Hunskaar et al., 1985). The same author showed that the doses of 5 mg/kg of morphine and 400 mg/kg of acetylsalicylic acid had a similar time-dependent effect on the immediate response to acute noxious stimuli (Hunskaar, 1987). Moreover, opioids are active in both phases of the test (Malmberg and Yaksh, 1993).

The results described in this paper demonstrate that subactive doses of acetylsalicylic acid and morphine, when given in combination, possess a significant analgesic activity in the hot-plate and formalin tests. Moreover, acetylsalicylic acid was able to potentiate the analgesic effect of 5 mg/kg of morphine, a dose which produced a significant increase in the %MPE when injected alone.

The combination of low doses of morphine and acetylsalicylic acid may be of interest from a therapeutic point of view, since the majority of drug combinations have secondary antinociceptive effects. Under our conditions, most of the unwanted reactions seen when a fully analgesic dose of both drugs is used may be avoided. Also, the potentiation of the effect of morphine by a low dose of acetylsalicylic acid may be beneficial in that it offers the chance of increasing the analgesic effect of other drugs without increasing the dosage. Some authors have shown that, using a wide range of dose combinations of acetylsalicylic acid and morphine, the two drugs have additive or potentiating effects depending on the dose used, while the degree of potentiation depends on the dose combination (Lopez-Munoz and Salazar, 1993).

The finding that naloxone blocked the potentiation of analgesia produced by the combination of the two drugs in the hot-plate test and in the first phase of the formalin test suggests that acetylsalicylic acid and morphine might exert their analgesic activity via a mechanism related to opioid receptor activation. In recent papers we hypothesized that the mechanism of action by which some NSAIDs exert

their analgesic action may involve the opioidergic pathways that in turn activate the serotonergic system (Pini et al., 1997a,b). This is supported by findings which indicate that serotonin (5-HT) takes part in the complex antinociceptive pathways, where it plays a pivotal role in nociception (Richardson, 1990).

Morphine has also been shown to act like acetylsalicylic acid on serotonergic pathways, increasing the concentration of 5-HT in the cerebral cortex and decreasing the number of 5-HT_2 receptors in the same area (Pini et al., 1997b). Some authors suggest that these effects are regionally selective: measurement of serotonin levels in brain areas after morphine (10 mg/kg) showed an increase in extracellular serotonin in the nucleus accumbens, amygdala, striatum, thalamus, hypothalamus and ventral hippocampus, but not in the other areas investigated and provided evidence that morphine acts by enhancing 5-HT levels in specific brain areas (Tao and Auerbach, 1995). Although the mechanism by which acetylsalicylic acid interacts with opioidergic and serotonergic systems has not yet been elucidated, data obtained in vitro in our laboratory have demonstrated that acetylsalicylic acid and morphine do not bind to opiate and 5-HT_2 receptors in the cerebral areas studied, while the two drugs increase serotonin concentrations in the same areas. This increase may explain the analgesic activity when the two drugs are given in combination.

An indirect increase in 5-HT levels in the CNS induced by acetylsalicylic acid and morphine has been observed previously. Groppetti et al. (1988) found a correlation between the increase in 5-HT in several areas of the brain and the antinociceptive activity of acetylsalicylic acid; Grauer et al. (1992) and Yoshida et al. (1993) demonstrated that morphine increases 5-HT levels in the CNS and observed a good correlation between the dose–response curves and time course for the morphine-induced increase in forebrain extracellular 5-HT levels and analgesia, as evaluated in the hot-plate test (Grauer et al., 1992). We confirm that the antinociceptive effects match the biochemical changes which suggests that the two results are possibly correlated. Naloxone, at the dose used in the present study, completely abolished the effect of acetylsalicylic acid and morphine given alone both in the hot-plate and in the formalin tests (Pini et al., 1997b).

We may conclude that the effect of acetylsalicylic acid + morphine takes place only when the two drugs are given in combination and when opiate receptors are blocked by naloxone, the antinociceptive effect disappeared in the hot-plate test and in the first phase of the formalin test.

Nevertheless, in this study naloxone did not exert full antagonism in the second phase of the formalin test. This difference cannot be easily explained on the basis of the present data, but the incomplete prevention of biochemical changes induced by the combination in the serotonin system (namely 5-HT_2 receptors) is in line with the result obtained in the formalin test. We can therefore hypothesize that the opioidergic influence in this second phase is of less importance than in the first phase, and/or that the combination of the drugs used exerts its activity through a more complex mechanism which differs partially from that of the two drugs administered alone. In the second phase of the formalin test there is an important action at a peripheral level where inflammatory processes play a pivotal role. Neurons of the dorsal horns of the spinal cord become activated and may contribute to the potentiation of the effect of morphine by acetylsalicylic acid in this phase. Other mechanisms seem to be involved at a central level because of the incomplete antagonism of the changes in 5-HT_2 receptors. Indeed, many findings indicate that the central antinociceptive system is a multi-step system, where presumably there is an altered balance between a number of different modulatory substances involving opioid and non-opioid-induced changes in pain perception (Björkman, 1995).

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