ELSEVIER

European Journal of Pharmacology 345 (1998) 233–245

ejp

Spinal and supraspinal antinociceptive action of dipyrone in formalin, capsaicin and glutamate tests. Study of the mechanism of action

Alessandra Beirith [a], Adair R.S. Santos [a], Ana L.S. Rodrigues [b], Tânia B. Creczynski-Pasa [c], João B. Calixto [a,*]

[a] *Department of Pharmacology, Centre of Biological Sciences, Federal University of Santa Catarina, Rua Ferreira Lima, 82, Florianópolis, SC, 88015-420, Brazil*

[b] *Department of Biochemistry, Centre of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88015-420, Brazil*

[c] *Department of Physiology, Centre of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88015-420, Brazil*

Received 21 April 1997; revised 30 December 1997; accepted 6 January 1998

Abstract

Dipyrone injected intraperitoneally (i.p.) or subplantarly into the mouse paw caused dose-related antinociception against the early and the late phases of formalin-induced licking, with mean ID_{50} values of 154.5 and 263.7 μmol/kg, and 2.6 and 1.2 μmol/paw, respectively. Given either by intracerebroventricular (i.c.v.) or by intrathecal (i.t.) routes, dipyrone produced a similar inhibition of both phases of the formalin-induced licking, with mean ID_{50} values of 0.4 and 1.3 μmol/site, and 0.4 and 0.9 μmol/site against the early and the late phase of the formalin response, respectively. Dipyrone, given by i.p., subplantar, i.t. or i.c.v. routes, caused dose-related antinociception of capsaicin-induced licking. The mean ID_{50} values were: 207.6 μmol/kg, 2.2 μmol/paw, 0.4 μmol/site and 0.14 μmol/site, respectively. In addition, dipyrone given i.p. caused a significant increase of the latency both in the hot-plate and the tail-flick assays. Dipyrone, given i.p., i.t. or i.c.v., reversed significantly the hyperalgesia caused by i.t. injection of glutamate, with mean ID_{50} values of 9 μmol/kg, 29 nmol/site and 94 nmol/site, respectively. The antinociception caused by dipyrone was not influenced by naloxone, L-arginine, phaclofen, glibenclamide, *p*-chlorophenylalanine methyl ester, pertussis toxin or by adrenal gland hormones, when assessed against the formalin assay. Dipyrone analgesic action was not secondary to its anti-inflammatory effect, nor was it associated with non-specific effects such as muscle relaxation or sedation actions of animals. Dipyrone at a higher concentration caused significant inhibition of [^{3}H]glutamate binding (37%) in cerebral cortical membranes from both mice and rats. However, dipyrone had no significant effect on brain constitutive neuronal nitric oxide synthase activity. It is concluded that dipyrone produces peripheral, spinal and supraspinal antinociception when assessed on formalin and capsaicin-induced pain as well as in glutamate-induced hyperalgesia in mice. Dipyrone antinociception seems unlikely to involve an interaction with the L-arginine-nitric oxide pathway, serotonin system, activation of G_i protein sensitive to pertussis toxin, interaction of ATP-sensitive K^+ channels, $GABA_B$ receptors, or the release of endogenous glucocorticoids. However, a modulatory effect on glutamate-induced hyperalgesia and, to a lesser extent, an interaction with glutamate binding sites, seems to account for its analgesic action. © 1998 Elsevier Science B.V.

Keywords: Dipyrone; Antinociception; Formalin test; Capsaicin test; Glutamate test; Hot-plate test; Tail-flick test; Nitric oxide (NO); Neurogenic pain; Glutamate binding; Pain; (Mouse)

1. Introduction

Dipyrone, a pirazolone derivative, has antipyretic, analgesic and antiinflammatory effects. It has been reported that dipyrone may exert its effects on inflammatory pain through inhibition of prostaglandin synthesis in both the periphery and the central nervous system (Abbate et al., 1990; Shimada et al., 1994), although its precise mechanism of action remains unclear and seems to be different from that of the classical non-steroidal antiinflammatory drugs (Lorenzetti and Ferreira, 1985; Engelhardt et al., 1995). It has been suggested that the antinociceptive action of dipyrone, as well as of other non-steroidal antiinflammatory drugs, is centrally mediated (Gelgor et al., 1992; for review see Tracey and Walker, 1995).

In recent years, it has been demonstrated that among the various neurotransmitters involved in nociceptive reac-

* Corresponding author. Tel.: +55-482-319491; fax: +55-482-224164; e-mail: calixto@ccb.ufsc.br

tions, the excitatory amino acid, glutamate, acting particularly on NMDA receptors, is of utmost importance to the transmission of both acute and chronic pain (for review see Dickenson, 1995). Furthermore, the activation of nitric oxide synthase with the consequent production of nitric oxide (NO) triggered by the NMDA receptor activation, seems to be involved in pain states (Mao et al., 1992; for review see Meller and Gebhart, 1993; Dickenson, 1995; Dray, 1995).

The antinociceptive action of dipyrone on inflammation-induced pain in the rat paw has been reported to be dependent on local participation of nitric oxide, as both the nitric oxide synthase inhibitor, N^G-methyl-L-arginine (L-NMMA), and methylene blue, an inhibitor of soluble guanylate cyclase, consistently prevent its analgesic effect (Duarte et al., 1992; Granados et al., 1995; Lorenzetti and Ferreira, 1996).

The aim of the present study was: (a) to evaluate the antinociceptive effect of dipyrone, given by several routes, on chemical and thermal models of nociception, as well as in the hyperalgesia caused by intrathecal injection of glutamate in mice, and (b) to investigate, by means of pharmacological as well as neurochemical approaches, possible mechanisms that may underlie its analgesic effects.

2. Methods

2.1. Animals

Male Swiss mice (25–35 g) and male Wistar rats (200–300 g), housed at 22 ± 2°C under a 12-h light/12-h dark cycle and with access to food and water ad libitum, were used. Experiments were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were only used once. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

2.2. Formalin test

The procedure used was essentially similar to that described previously (Corrêa and Calixto, 1993; Vaz et al., 1996): 20 μl of 2.5% formalin solution (0.92% of formaldehyde), made up in phosphate buffer solution (PBS; concentration: NaCl 137 mM, KCl 2.7 mM and phosphate buffer 10 mM), was injected subcutaneously (s.c.) under the surface of the right hindpaw. Two mice (control and treated) were observed simultaneously from 0 to 30 min following formalin injection. The early phase of the nociceptive response normally peaked 0 to 5 min after formalin injection and the late phase 15 to 30 min after formalin injection, representing the neurogenic and inflammatory pain responses, respectively. Animals were treated with dipyrone intraperitoneally (i.p., 90–720 μmol/kg) 30 min before the formalin injection, or subplantarly, co-injected in association with formalin (0.1–30 μmol/paw). Other groups of animals were treated with dipyrone intracerebroventricularly (i.c.v., 0.09–1.8 μmol/site) or intrathecally (i.t., 0.3–3.0 μmol/site) as described previously (Hylden and Wilcox, 1980; Laursen and Belknap, 1986; Vaz et al., 1996), 10 min before formalin injection. Following intraplantar injection of formalin, the animals were immediately placed in a glass cylinder 20 cm in diameter, and the time spent licking the injected paw, was timed with a chronometer and considered as indicative of pain. Control animals received a similar volume of 0.9% of NaCl solution systemically (10 ml/kg), subplantarly (20 μl/paw), i.c.v. (5 μl/site) or i.t. (5 μl/site).

In order to investigate whether the antinociceptive activity of dipyrone in formalin-induced pain was associated with anti-oedematogenic activity, we measured the paw oedema by comparing the difference in weight of the formalin-treated paw and the weight of the control paw (treated with saline). For this purpose, animals were killed 30 min after formalin injection by cervical dislocation, and the paw was cut at the knee joint and weighed on an analytical balance.

2.3. Capsaicin-induced pain

In an attempt to provide more direct evidence concerning a possible analgesic effect of dipyrone on neurogenic pain, we also investigated whether this drug antagonised capsaicin-induced pain in the mouse paw. The procedure used was similar to that described previously (Corrêa et al., 1996; Santos and Calixto, 1997). Before testing, the animals were placed individually in transparent glass cylinders 20 cm in diameter, serving as observation chambers. Following the adaptation period, 20 μl of capsaicin (1.6 μg/paw made in PBS) was injected under the skin of the dorsal surface of the right hindpaw. The contralateral paw received a similar volume of saline. The animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of pain. Animals were treated with dipyrone systemically (30–360 μmol/kg), subplantarly (0.1–30 μmol/paw), co-injected in association with capsaicin, or by i.c.v. (0.09–1.8 μmol/site) or i.t. (0.3–3.0 μmol/site) routes as described before. Control animals received a similar volume of 0.9% of NaCl solution systemically (10 ml/kg), subplantarly (20 μl/paw), i.c.v. (5 μl/site) or i.t. (5 μl/site).

2.4. Glutamate-induced hyperalgesia

To test the hypothesis that the excitatory amino acids were involved in the dipyrone antinociception, we assessed

the effect of dipyrone given by different routes on the hyperalgesic responses caused by spinally administered glutamate (100 nmol/site, i.t.) in mice in the hot plate test. Experiments were carried out as described by Aanonsen and Wilcox (1987) with minor modifications, involved control animals (0.9% NaCl solution-treated) or mice pre-treated with dipyrone (3–30 μmol/kg, i.p., 9–90 nmol/site, i.t. or 30–300 nmol/site, i.c.v.) 30 and 10 min prior, with the exception of those pre-treated by the i.t. route in which case the dipyrone was co-administered with glutamate. The pain response was measured on the hot-plate apparatus (Ugo Basile, model-DS 37) maintained at 50 ± 1°C as described in item 2.6. The maximal hyperalgesic response caused by i.t. injection of glutamate was observed for 60 min after the injection. This time was used in all future experiments. A cut-off of 30 s was used for the hot plate. The maximal percentage of inhibition (MPI) of glutamate-induced hyperalgesia was calculated as follows:

$$\%\,\mathrm{MPI} = \frac{\text{postdrug} - \text{predrug}}{30 - \text{predrug}} \times 100$$

2.5. Analysis of the antinociceptive mechanism of action of dipyrone

To investigate the participation of the opioid system in the antinociceptive effect of dipyrone, animals were pre-treated with naloxone (an antagonist of opioid receptors, 13.7 μmol/kg, i.p.), 15 min before the administration of dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.). The other groups of animals received dipyrone, morphine, naloxone or saline 30 min before the formalin injection (Vaz et al., 1996). To assess the possible participation of G_i protein (sensitive to pertussis toxin) in the antinociceptive action of dipyrone, animals were pre-treated with pertussis toxin (1.0 μg/site, i.c.v.) 7 days before the administration of dipyrone (540 μmol/kg, i.p.) or morphine (13.3 μmol/kg, s.c.), used as positive control. Other groups of animals were treated with saline (0.9% NaCl solution, 5 μl/site, i.c.v), and 7 days later received dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or the vehicle (0.9% NaCl solution, 10 ml/kg, i.p.), 30 min before the formalin injection (Sánchez-Blázquez and Garzón, 1991).

To assess the possible participation of the γ-aminobutyric acid (GABAergic) system, animals were treated with dipyrone (540 μmol/kg, i.p.) or with baclofen (4.6 μmol/kg, i.p.) (a selective $GABA_B$ receptor agonist) 30 min prior to the injection of formalin (Vaz et al., 1996). The animals received phaclofen (40 μmol/kg, i.p.), a selective $GABA_B$ receptor antagonist, 10 min before the administration of dipyrone, baclofen or saline (0.9% NaCl solution, 10 ml/kg, i.p.).

In a separate series of experiments, we also investigated the possible participation of the nitric oxide-L-arginine pathway in the analgesic effect caused by dipyrone. To this end, animals were pre-treated with L-arginine (3444 μmol/kg, i.p.), and after 15 min they received dipyrone (540 μmol/kg, i.p.), N^G-nitro-L-arginine (a nitric oxide synthase inhibitor, L-NOARG, 342 μmol/kg, i.p.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.). The algesic responses in the early and the second phase of the formalin test were recorded 30 min after administration of dipyrone, L-NOARG or saline. Other groups of animals received only dipyrone (540 μmol/kg, i.p.), L-NOARG (342 μmol/kg, i.p.), L-arginine (3444 μmol/kg, i.p.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.) 30 min before formalin injection (Santos et al., 1995; Vaz et al., 1996).

To investigate the possible contribution of serotonin to the analgesic effect of dipyrone, animals were pre-treated with DL-*p*-chlorophenylalanine methyl ester hydrochloride (an inhibitor of serotonin synthesis, 399.8 μmol/kg, i.p. once a day for 4 consecutive days), prior to the administration of dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.). The pain response caused by subplantar formalin injection was analysed 30 min after drug administration (Santos et al., 1995; Vaz et al., 1996). Other groups of mice received only dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.) 30 min before formalin injection.

We also investigated the possible role of the K_{ATP} channel in the analgesic effect caused by dipyrone. For this purpose, animals were pre-treated with glibenclamide (81 nmol/site, i.c.v.) (a K_{ATP} channel blocker), and after 15 min they received dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.) (Raffa and Martinez, 1995). The algesic responses caused by formalin were recorded 30 min after administration of dipyrone, morphine or saline. Other groups of animals received saline (5 μl/site, i.c.v.), 15 min prior to the administration of dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.) 30 min before formalin injection.

To assess the possible participation of endogenous glucocorticoids in the antinociceptive effect caused by dipyrone, animals were anaesthetised with 2,2,2-tribromoethanol (0.25 g/kg, i.p.) and both adrenal glands were removed through a dorsal incision, as described previously by Vaz et al. (1996). After surgery, the animals were returned to their cages, with free access to food and drink, but the water was replaced by saline (0.9% NaCl solution) to maintain a physiological plasma sodium concentration. Another group of animals was sham-operated and allowed free access to water and food. After one week, the animals received dipyrone (540 μmol/kg, i.p.) or 0.9% NaCl solution (10 ml/kg, i.p.), 30 min before formalin injection. The sham-operated animals were used as controls.

2.6. Hot-plate test

The hot-plate test was used to measure the response latencies according to the method described previously by Eddy and Leimbach (1953), with minor modifications. In these experiments, the hot-plate (Ugo Basile, model-DS 37) was maintained at 56 ± 1°C. Animals were placed into a glass cylinder with 24-cm diameter on the heated surface, and the time between placement and shaking or licking of the paws or jumping was recorded as the index of response latency. An automatic 30-s cut-off was used to prevent tissue damage. Each animal was tested before administration of drugs in order to obtain the baseline. Control animals (0.9% NaCl solution, 10 ml/kg, i.p.), or mice pre-treated with dipyrone (600 μmol/kg, i.p.) 30 min earlier, were placed into a glass cylinder with 24-cm diameter on the heated surface. Other groups of animals were treated with morphine (26.6 μmol/kg, s.c., 30 min prior).

2.7. Tail-flick test

A radiant heat tail-flick analgesiometer was used to measure response latencies according to the method described previously by D'Amour and Smith (1941), with minor modifications. Animals responded to a focused heat stimulus by flicking or removing their tail, exposing a photocell in the apparatus immediately below it. The reaction time was recorded for control mice (0.9% NaCl solution, 10 ml/kg, i.p.) and for animals pre-treated with dipyrone (600 μmol/kg, i.p.) or with morphine (26.6 μmol/kg, s.c.) 30 min before. An automatic 20-s cut-off was used to minimise tissue damage. Animals were selected 24 h previously on the basis of their reactivity in the test. To determine the baseline, each animal was tested before administration of drugs.

2.8. Measurement of motor performance

In order to evaluate possible non-specific muscle relaxant or sedative effects of dipyrone, mice were tested on the rota-rod (Rosland et al., 1990). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 revolutions per minute. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 60 s. Animals were treated with dipyrone (600 μmol/kg, i.p.) or with the same volume of 0.9% NaCl solution (10 ml/kg, i.p.) 30 min before being tested. The results are expressed as the time (s) for which animals remained on the rota-rod. The cut-off time used was 60 s.

2.9. Brain constitutive neuronal NO synthase preparation and enzyme activity

The procedure used was described previously by Knowles et al. (1990). The cerebella of five rats were homogenised with a tissue tearer, in a buffer (1:5 w/v) containing 50 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10 mg/ml soy bean trypsin inhibitor, 2 mg/ml aprotinin and 320 mM sucrose at 4°C. The homogenate was centrifuged at 4°C at 10 000 × *g* for 20 min and the supernatant was divided into aliquots and frozen at −70°C. Constitutive neuronal NO synthase activity persisted for several weeks after preparation.

NO synthase activity was measured as the ability of tissue homogenates to convert L-[^{3}H]arginine to L-[^{3}H]citrulline. The procedure used was originally described by Bredt and Snyder (1990) and modified by Wolff and Datto (1992). The standard reaction medium contained 50 mM KH_2PO_4, pH 7.2, 1.2 mM $MgCl_2$, 0.25 mM $CaCl_2$, 60 mM L-valine, 1.2 mM L-citrulline, L-[^{3}H]arginine, 1.6 mCi/ml (not considering the endogenous concentration of L-arginine), 1 mM dithiothreitol, 4 μM flavine adenine dinucleotide, 4 μM flavine mononucleotide, 10 μM tetrahydrobiopterin and 120 μM nicotinamide adenine dinucleotide phosphate (reduced form), in the presence or in the absence of dipyrone. The reactions were initiated by the addition of enzyme preparation (1.3 mg/ml), since no pre-incubation was necessary. After 1 h of incubation at room temperature, the reactions were stopped by mixing with the weak cationic exchange resin, AG 50W-X8 (10 g per 100 ml in water). The samples were centrifuged at 10 000 × *g* for 5 min and aliquots from the supernatant were mixed with the scintillation liquid and counted. The constitutive neuronal NO synthase activity was obtained by subtraction of the calcium-independent activity (measured in the presence of 1 mM ethylene glycol tetraacetic acid).

2.10. [^{3}H]glutamate binding assay

Cerebral cortices obtained from both mice and rats (killed by decapitation) were dissected and homogenised in 20 volumes of ice-cold 0.32 M sucrose containing 10 mM Tris–HCl buffer, pH 7.4, and 1 mM $MgCl_2$. The homogenate was centrifuged at 1000 × *g*, and the pellet was re-homogenised and centrifuged again. The second pellet was discarded, and both supernatants were pooled and centrifuged at 27 000 × *g* for 15 min. The resulting pellet was dissolved in 1 mM Tris–HCl, pH 7.4, for 30 min. The pellet was washed three times in 10 mM Tris–HCl buffer, pH 7.4, at 27 000 × *g* for 15 min (Souza and Ramírez, 1991). The final pellet was diluted in 10 mM Tris–HCl and frozen at −70°C until use. On the day of the experiment, the membranes were thawed and incubated with

0.04% Triton X-100 at 37°C for 30 min and were then washed three times in 10 mM Tris–HCl buffer, pH 7.4, at 27 000 × *g* for 15 min. The final pellet was resuspended in 10 mM Tris–HCl and the suspensions were assayed for [^{3}H]glutamate binding. Protein concentration was determined in aliquots of this final suspension by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Assays of [^{3}H]glutamate binding were carried out in triplicate in a total volume of 0.5 ml containing 0.1 ml membrane (0.2–0.3 mg protein), 50 mM Tris–HCl pH 7.4, 40 nM radioactive ligand ([^{3}H]glutamate, 53 Ci/mmol), in the presence or absence of dipyrone (concentration in the range 10–1000 μM). Unspecific binding was assayed similarly, except that 40 μM non-radioactive glutamate (displacer) was added to the incubation medium. Centrifugation at 12 000 × *g* for 25 min was used to separate [^{3}H]glutamate not bound to membranes. The supernatant was discarded, and the walls of the Eppendorf tubes and the surfaces of the pellets were quickly and carefully rinsed with cold deionized water, followed by processing for radioactivity. Specific binding was calculated as the difference between binding values in the absence and the presence of the displacer. The results represent the means of three independent experiments.

2.11. Drugs

The following substances were used: formalin, morphine hydrochloride (Merck, Darmstadt, Germany), dipyrone, L-glutamic acid, DL-*p*-chlorophenylalanine methyl ester hydrochloride, pertussis toxin, 2,2,2-tribromoethanol, L-arginine, N^G-nitro-L-arginine (Sigma Chemical, St. Louis, USA), naloxone hydrochloride (Dupont, Garden City, USA), capsaicin (Calbiochen, San Diego, CA, USA), glibenclamide, baclofen and phaclofen (Research Biochemicals International, Natick, MA, USA). [^{3}H]glutamate (53 Ci/mmol) and L-[^{3}H]arginine (64 Ci/mmol) were purchased from Amersham International, UK. Drugs were prepared just before use in 0.9% w/v of NaCl, with the exception of capsaicin which was prepared in absolute ethanol. The final concentration of ethanol did not exceed 5% and did not cause any effect 'per se'. In the neurochemical studies, drugs were dissolved in deionized water.

2.12. Statistical analysis

The results are presented as means ± S.E.M., except the ID_{50} values (i.e., the dose or the concentration of drugs reducing the pain responses by 50% relative to the control value) which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance of differences between groups was evaluated by means of analysis of variance followed by Dunnett's multiple comparison test or by Newmann–Keuls' test when appropriate. P values less than 0.05 ($P < 0.05$) were considered as indicative of significance. The ID_{50} values were determined by graphic interpolation from individual experiments.

3. Results

3.1. Formalin-induced pain

The results of Fig. 1 (A and B) show that dipyrone (90–720 μmol/kg, i.p.) caused a significant dose-related inhibition of the neurogenic (0 to 5 min) and the inflammatory phase (15 to 30 min) of the formalin-induced licking. The calculated mean ID_{50} values for these effects were 154.5 (99.9–238.8) and 263.7 (234.0–296.9) μmol/kg, respectively (Table 1). Dipyrone was significantly more active to inhibit the inflammatory phase than the neurogenic phase of the formalin response. The maximal inhibitions were 74 ± 2% and 91 ± 1%, respectively (Table 1). However, dipyrone had no significant effect on the paw oedema associated with the inflammatory phase of the formalin test (results not shown).

When co-injected subplantarly in association with formalin, dipyrone (0.1–30 μmol/paw) produced a dose-related inhibition of both the neurogenic and the inflammatory phase of the formalin test, its effect being more potent

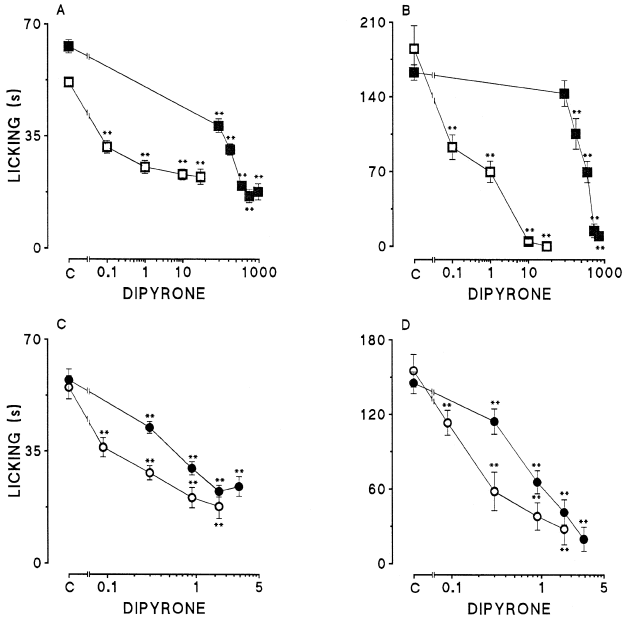

Fig. 1. Effects of dipyrone given intraperitoneally (-■-, 90–720 μmol/kg), subplantarly (-□-, 0.1–30 μmol/paw), intracerebroventricularly (-○-, 0.09–1.8 μmol/site) and intrathecally (-●-, 0.3–3.0) against the early (panel A and C) and the second phase (panel B and D) of formalin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min) and the second phase (15–30 min) after subplantar injection of formalin into the hindpaw. Each point represents the mean ± S.E.M. for 6–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, ** $P < 0.01$. In some cases, the S.E.M. are hidden within the symbols.

Table 1
The mean ID_{50}s and maximal inhibition values for the antinociceptive action of dipyrone in formalin- and capsaicin-induced licking and glutamate-induced hyperalgesia in mice

Dipyrone	Early phase		Late phase	
	ID_{50}[a]	MI (%)[b]	ID_{50}[a]	MI (%)[b]
	Formalin			
Intraperitoneal (μmol/kg)	154.5 (99.9–238.8)	74 ± 2	263.7 (234.3–296.9)	91 ± 1
Subplantar (μmol/paw)	2.6 (0.8–8.4)	57 ± 5	1.2 (0.2–9.2)	100
Intracerebroventricular (μmol/site)	0.4 (0.3–0.7)	68 ± 7	0.4 (0.3–0.5)	82 ± 8
Intrathecal (μmol/site)	1.3 (0.9–1.8)	61 ± 3	0.9 (0.6–1.4)	86 ± 7
	Capsaicin			
Intraperitoneal (μmol/kg)	207.6 (179.5–240.0)			70 ± 8
Subplantar (μmol/paw)	2.2 (0.5–9.2)			66 ± 8
Intracerebroventricular (μmol/site)	0.14 (0.11–0.19)			80 ± 5
Intrathecal (μmol/site)	0.4 (0.3–0.6)			68 ± 4
	Glutamate			
Intraperitoneal (μmol/kg)	9.0 (6.8–11.9)			100
Intrathecal (nmol/site)	28.7 (24.3–34.0)			100
Intracerebroventricular (nmol/site)	93.91 (70.8–124.8)			100

Each group represents the mean for 6 to 10 animals. [a]ID_{50}s with their respective 95% confidence limits, [b]Maximal inhibitions.

(about 2-fold) and efficacious against the second phase (Fig. 1A and B). The calculated mean ID_{50} values and the maximal inhibitions of the early and the late phase were 2.6 (0.8–8.4) and 1.2 (0.2–9.2) μmol/paw and 57 ± 5% and 100%, respectively (Table 1).

Given i.c.v. (0.09–1.8 μmol/site) dipyrone produced dose-dependent and equipotent inhibition of both phases of the formalin-induced licking (Fig. 1 C and D). The calculated mean ID_{50} values for these effects (μmol/site) were 0.4 (0.3–0.7) and 0.4 (0.3–0.5), against the early and the late phase of the formalin response, respectively. The calculated maximal inhibitions of the early and the late phase were 68 ± 7% and 82 ± 8%, respectively (Table 1).

Given i.t. (0.3–3.0 μmol/site) dipyrone produced dose-dependent and equipotent inhibition of both phases of the formalin-induced licking (Fig. 1 C and D). The calculated mean ID_{50} values for these effects (μmol/site) were 1.3 (0.9–1.8) and 0.9 (0.6–1.4), against the early and the late phase of the formalin response, respectively. The maximal inhibitions of the early and the late phase were 61 ± 3% and 86 ± 7%, respectively (Table 1).

3.2. Capsaicin-induced pain

The results of Fig. 2 (A and B) show that dipyrone, given systemically (30–360 μmol/kg, i.p.), subplantarly (0.1–30 μmol/paw), i.t. (0.3–3.0 μmol/site) or i.c.v. (0.09–1.8 μmol/site) route, dose dependently inhibited capsaicin-induced licking. The calculated mean ID_{50} values and the maximal inhibitions were: 207.6 (179.5–240.0) μmol/kg, 2.2 (0.5–9.2) μmol/paw, 0.4 (0.3–0.6) μmol/site and 0.14 (0.11–0.19) μmol/site and 70 ± 8%, 66 ± 8%, 68 ± 4% and 80 ± 5%, respectively (Table 1).

3.3. Glutamate-induced hyperalgesia

Fig. 3 (A, B and C) shows that dipyrone, given systemically (3–30 μmol/kg, i.p.), i.c.v. (30–300 nmol/site) or i.t. (9–90 nmol/site), dose dependently inhibited glutamate-induced hyperalgesia. The calculated mean ID_{50} values (estimated at 5 min) were: 9.0 (6.8–11.9) μmol/kg, 93.9 (70.8–124.8) nmol/site and 28.7 (24.2–34.0) nmol/site, respectively and the maximal inhibitions were 100% (Table 1). Given alone, dipyrone, over the same range of doses effective to inhibit glutamate-induced hyperalgesia, had no effect in the hot-plate assay (results not shown).

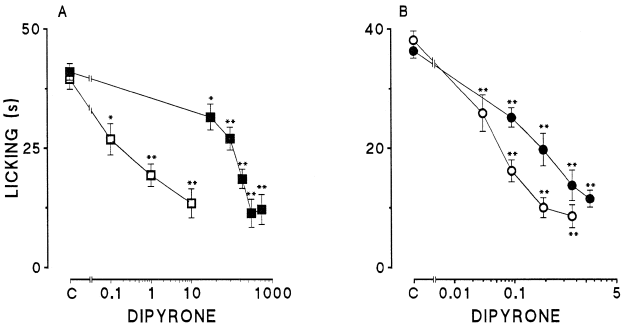

Fig. 2. Effects of dipyrone given intraperitoneally (-■-, 30–360 μmol/kg), subplantarly (-□-, 0.1–30 μmol/paw) (panel A), intracerebroventricularly (-○-, 0.09–1.8 μmol/site) and intrathecally (-●-, 0.3–3.0 μmol/site) (panel B) against capsaicin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the 0–5 min range after subplantar injection of capsaicin into the hindpaw. Each point represents the mean ± S.E.M. for 8–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, $* P < 0.05$ and $** P < 0.01$.

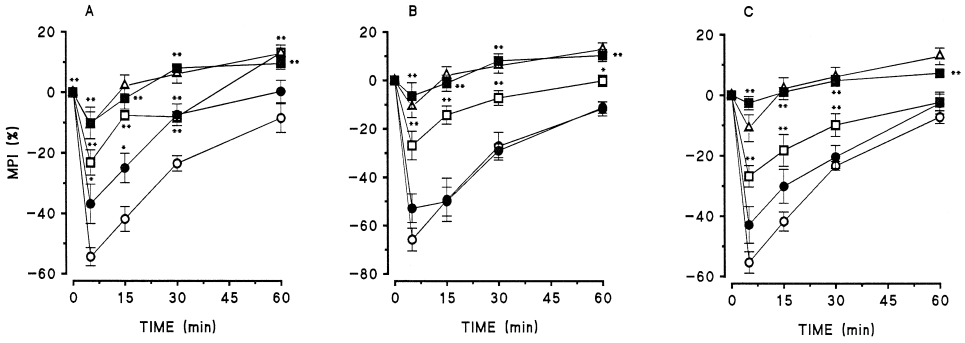

Fig. 3. Effects of dipyrone given intraperitoneally (-○- = vehicle, -●- = 3 μmol/kg, -□- = 9 μmol/kg, -■- = 30 μmol/kg and -△- vehicle i.p. + vehicle i.t.) (panel A), intracerebroventricularly (-○- = vehicle, -●- = 30 nmol/site, -□- = 90 nmol/site, -■- = 300 nmol/site and -△- vehicle i.c.v. + vehicle i.t.) (panel B) and intrathecally (-○- = vehicle, -●- = 9 nmol/site, -□- = 30 nmol/site, -■- = 90 nmol/site, and -△-vehicle i.t.) (panel C) against glutamate-induced hyperalgesia in mice. The latency (mean ± S.E.M.) in the hot plate was measured at 5–60 min after intrathecal injection of glutamate. Each point represents the mean ± S.E.M. for 6–8 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, $* P < 0.05$, $** P < 0.01$. In some cases, the S.E.M. are hidden within the symbols.

3.4. Analysis of the antinociceptive mechanism of action of dipyrone

The results of Fig. 4 (A and B) show that the pre-treatment of animals with naloxone (13.7 μmol/kg, i.p.), 15 min before injection of morphine (13.3 μmol/kg, s.c.), largely reversed the antinociception caused by morphine when analysed for both phases of the formalin-induced licking, leaving the antinociceptive effect of dipyrone (540 μmol/kg, i.p.) unaffected. Treatment of the animals with

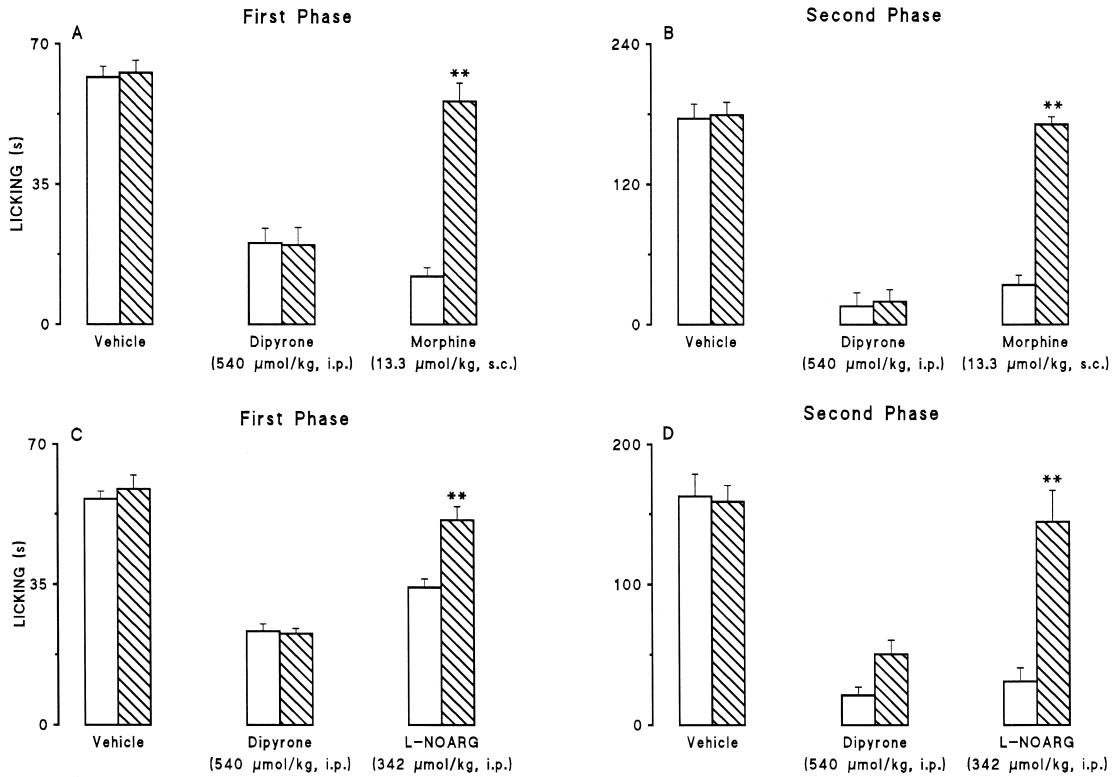

Fig. 4. Effects of pretreatment of animals with naloxone (13.7 μmol/kg, i.p., upper panels) or L-arginine (3444 μmol/kg, i.p., lower panels) 20 min before injection of dipyrone (540 μmol/kg, i.p.), morphine (13.3 μmol/kg, s.c.) or L-NOARG (342 μmol/kg, i.p.), against the first (panels A and C) and the second phase (panels B and D) of formalin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min) and the second phase (15–30 min) after subplantar injection of formalin into the hindpaw. Each column represents the mean for 8–10 animals and the vertical lines indicate the S.E.M. The open columns represent the control values and the hatched columns represent the treatment with naloxone or L-argine, (upper and lower panels, repectively). The asterisks denote the significance levels when compared with control groups. Significantly different from controls, $** P < 0.01$.

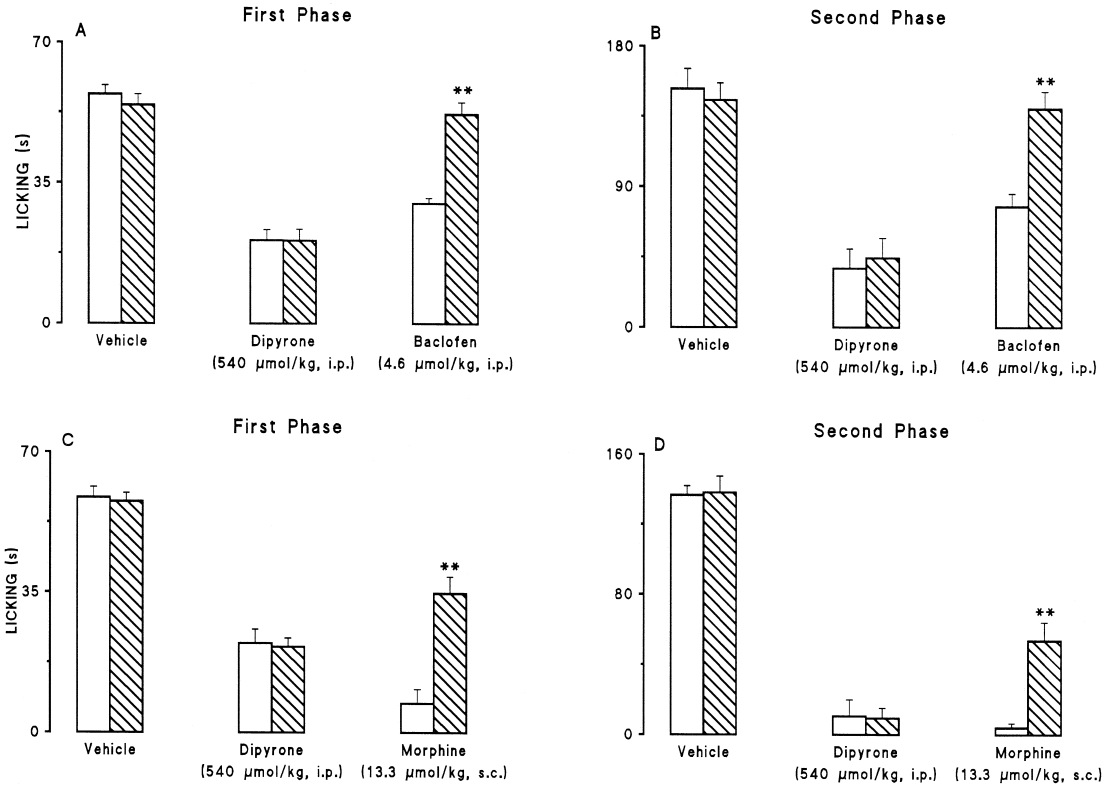

Fig. 5. Effects of pretreatment of animals with phaclofen (40 μmol/kg, i.p., upper panels) or glibenclamide (81 nmol/kg, i.c.v., lower panels) 20 min before injection of dipyrone (540 μmol/kg, i.p.), baclofen (4.6 μmol/kg, i.p.) or morphine (13.3 μmol/kg, s.c.), against the first (panels A and C) and the second phase (panels B and D) of formalin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min) and the second phase (15–30 min) after subplantar injection of formalin into the hindpaw. Each column represents the mean for 8–10 animals and the vertical lines indicate the S.E.M. The open columns represent the control values and the hatched columns represent treatment with phaclofen or glibencamide (upper and lower panels, respectively). The asterisks denote the significance levels when compared with control groups. Significantly different from controls, $** P < 0.01$.

L-NOARG (342 μmol/kg, i.p.), 30 min prior, produced marked antinociception against both phases of the formalin test, an effect which was almost completely reversed by L-arginine (3444 μmol/kg, i.p.) given 15 min prior to drug injections. However, L-arginine failed to significantly affect the antinociception caused by dipyrone (540 μmol/kg, i.p.) (Fig. 4C and D).

Previous treatment of the animals with phaclofen (40 μmol/kg, i.p.), 15 min before, significantly reversed the antinociception caused by baclofen (4.6 μmol/kg, i.p.), but did not significantly change the antinociceptive action caused by dipyrone (540 μmol/kg, i.p.) when analysed against both phases of the formalin test (Fig. 5A and B).

The K_{ATP} channel blocker, glibenclamide (81 nmol/site, i.c.v.), given 15 min beforehand, did not significantly modify the antinociceptive effect caused by dipyrone (540 μmol/kg, i.p.) against both phases of formalin-induced nociception (Fig. 5C and D). However, under the same conditions, glibenclamide significantly antagonised the antinociception caused by morphine (13.3 μmol/kg, s.c.) in the formalin test.

The results of Fig. 6 (A and B) show that pre-treatment of the animals with DL-*p*-chlorophenylalanine methyl ester hydrochloride (399.8 μmol/kg, i.p., once a day for 4 days), reversed the antinociceptive effect of morphine (13.3 μmol/kg, s.c.), but did not affect the antinociceptive effect of dipyrone (540 μmol/kg, i.p.) when tested against both phases of formalin-induced pain. The i.c.v. administration of pertussis toxin (1.0 μg/site, 7 days prior), an inactivator of G_i protein, caused a significant inhibition of morphine (13.3 μmol/kg, s.c.)-induced antinociception, while it had no significant effect on the analgesic action of dipyrone (540 μmol/kg, i.p.) when tested against both phases of formalin-induced pain (Fig. 6C and D).

Bilateral adrenalectomy of the animals, performed one week prior to experiments, did not interfere significantly with dipyrone (540 μmol/kg, i.p.)-induced antinociception when assessed in the formalin test (results not show).

3.5. Hot-plate and tail-flick tests

The results summarised in Table 2 show that dipyrone (up to 600 μmol/kg, i.p.), given 30 min prior, caused a significant increase of the latency response in both the hot-plate and the tail-flick assays. Under similar conditions, morphine (26.6 μmol/kg, s.c.), used as a reference

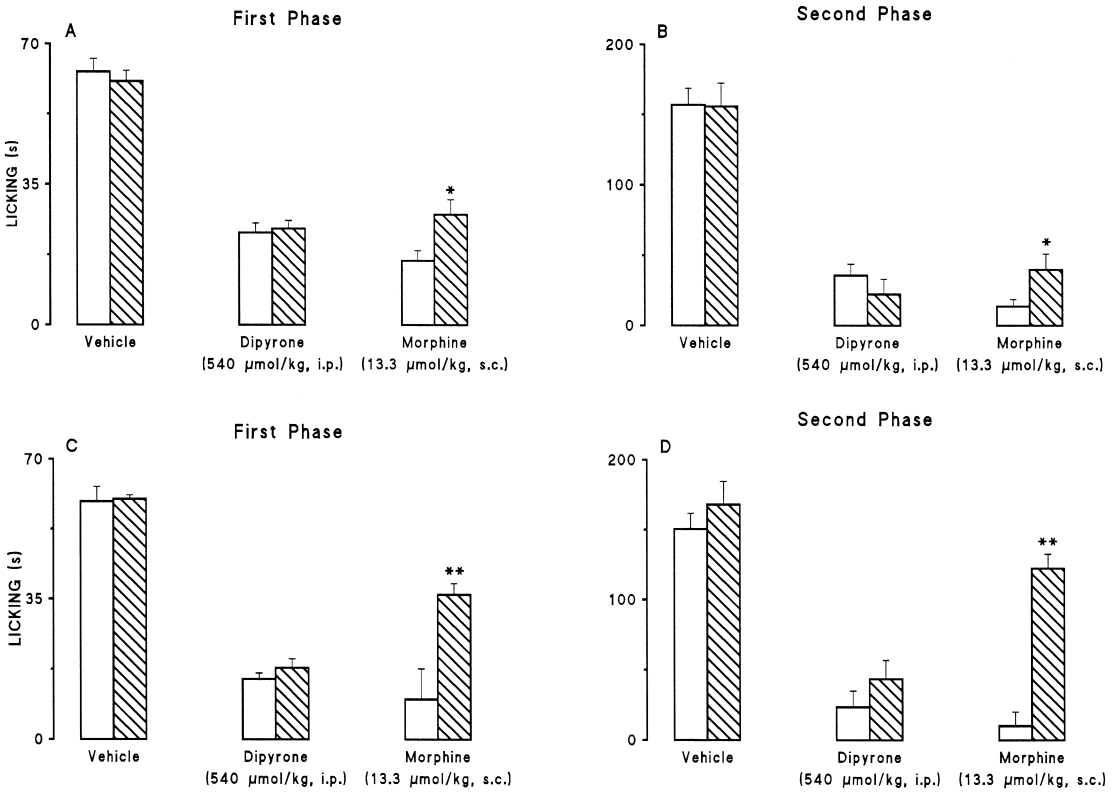

Fig. 6. Effects of pretreatment of animals with PCPA (399.8 μmol/kg, i.p., once a day for 4 days, upper panels) or pertussis toxin (1 μg/site 7 days before, lower panels) before injection of dipyrone (540 μmol/kg, i.p.) or morphine (13.3 μmol/kg, s.c.), against the first (panel A and C) and the second phase (panel B and D) of formalin-induced licking in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min) and the second phase (15–30 min) after subplantar injection of formalin into the hindpaw. Each column represents the mean for 8–10 animals and the vertical lines indicate the S.E.M. The open columns represent the control values and the hatched columns represent treatment with PCPA or pertussis toxin (upper and lower panels, respectively). The asterisks denote the significance levels when compared with control groups. Significantly different from controls, $* P < 0.05$ and $** P < 0.01$.

drug and given 30 min before, caused a significant and marked analgesic effect in both models.

3.6. Rota-rod test

Dipyrone (600 μmol/kg), given i.p., 30 min beforehand, did not significantly affect the motor response of the animals. The control response in the rota-rod test was 60 s vs. 60 s in the presence of dipyrone ($n = 8$).

Table 2
Analgesic effect of morphine (s.c.) and dipyrone (i.p.) in the hot plate and tail flick tests in mice

Drugs	Dose (μmol/kg)	Latency (s)	
		Tail-flick	Hot-plate
Control	0	4.9 ± 0.4	5.2 ± 0.5
Morphine	26.6	19.4 ± 0.6[a]	23.9 ± 1.5[a]
Dipyrone	600	11.8 ± 1.6[a]	9.1 ± 0.7[a]

Each group represents means ± S.E.M. for 6 to 10 animals. [a] $P < 0.01$ when compared to control value.

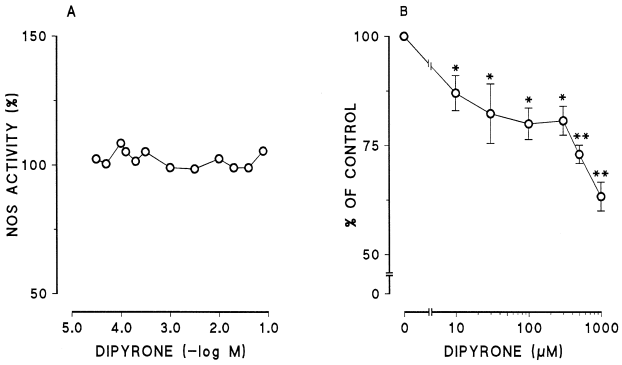

Fig. 7. Effect of increasing concentrations of dipyrone on constitutive neuronal NO synthase (panel A). Activity was measured with L-[^{3}H]arginine as described in Methods. The reaction was initiated by the addition of the constitutive neuronal NO synthase preparation (1.3 mg/ml). 100% of constitutive neuronal NO synthase activity is 0.5 ± 0.05 nmol/mg per min. The results are the means of 4 experiments and each experiment was carried out in duplicate. Effects of increasing concentrations of dipyrone (10–1000 μM) on [^{3}H]glutamate binding in mice cerebral cortex membranes (panel B). Each point represents the mean of three independent experiments (each experiment was carried out in triplicate) and the vertical lines indicate the S.E.M. The asterisks denote the significance levels when compared with control group, $* P < 0.05$ and $** P < 0.01$.

3.7. Brain NO synthase activity

Since treatment of the animals with L-arginine had no influence on the antinociceptive effect of dipyrone, we tested the influence of dipyrone directly on constitutive neuronal NO synthase activity. Dipyrone (30–90 μM) had no significant effect on constitutive neuronal NO synthase activity (Fig. 7A).

3.8. Effect of dipyrone on [^{3}H]glutamate binding

The results depicted in Fig. 7 (B) show that dipyrone (10–1000 μM) produced a modest but, significant inhibition of [^{3}H]glutamate binding in cerebral cortical membranes of mice. The maximal percentage inhibition (37 $\pm$ 3%) was observed at 1000 μM dipyrone. Dipyrone also caused a very similar inhibition of [^{3}H]glutamate binding in cerebral cortical membranes obtained from rats (results not shown).

4. Discussion

The results presented here demonstrate that dipyrone administered by the intraperitoneal, subcutaneous, spinal or supraspinal route elicited a significant and dose-dependent antinociception in mice, when assessed in two behavioural models of chemical pain, formalin- and capsaicin-induced licking. Interestingly, dipyrone, given intraperitoneally at higher doses, caused a discrete but significant latency increase in two algesiometer models of pain, the hot-plate and tail-flick tests. Furthermore, at lower doses given i.p., i.t. or i.c.v., dipyrone caused a dose-related attenuation of glutamate-induced hyperalgesia in mice. These findings extend previous results from the literature (Lorenzetti and Ferreira, 1985; López-Muñoz et al., 1994) and indicate that dipyrone, like morphine, but unlike most of the aspirin-like drugs, seems to have a direct effect on pain transmission.

Also relevant are the results showing that dipyrone exhibited almost the same potency at the ID_{50} levels for inhibiting the neurogenic (first phase) and the inflammatory (second phase) of the formalin-induced algesic response, in spite of being significantly more efficacious against the second phase of the formalin response. However, depending on the route of administration used, dipyrone was equipotent to prevent the nociception induced by subplantar injection of either formalin or capsaicin. At the ID_{50} level, dipyrone was about 13 to 541-fold less potent than morphine, but about 5 to 10-fold more potent than aspirin and acetaminophen, depending on the route of administration used (Vaz et al., 1996).

The potent and concentration-dependent antinociception elicited by dipyrone, given subplantarly by the spinal and supraspinal routes, against formalin and capsaicin-induced pain, is particularly relevant, as most of the non-steroidal antiinflammatory drugs tested were reported to be largely ineffective to prevent the neurogenic pain response induced by either formalin (first phase) or capsaicin (Shibata et al., 1989; Malmberg and Yaksh, 1992; Corrêa and Calixto, 1993; Vaz et al., 1996). Such findings strongly suggest that dipyrone has an additional and interesting mechanism of analgesic action beyond its well-known ability to inhibit the cyclo-oxygenase products derived from the arachidonic acid pathway (Ferreira et al., 1978; Abbate et al., 1990; Shimada et al., 1994).

The antinociception elicited by dipyrone and now reported upon is unlikely to be secondary to its reported anti-inflammatory property as regardless of ranges of doses or routes of administration tested, dipyrone did not inhibit the paw oedema formation associated with the later phase of the formalin-induced nociception. We and others have reported that formalin-induced paw oedema involves the release of several mediators, such as kinins, prostaglandins, excitatory amino acids and nitric oxide (Haley et al., 1990; Coderre and Melzack, 1992; Yamamoto and Yaksh, 1992a,b; Corrêa and Calixto, 1993; Corrêa et al., 1996). In contrast, tachykinins acting through NK_1, NK_2 or NK_3 receptors seem not to be involved in formalin-induced oedema formation (Santos and Calixto, 1997). Also, the antinociception caused by dipyrone is unlikely to be secondary to its depressant and/or non-specific depressant central effects as revealed by the lack of any detectable result in the rota-rod test.

The mechanism by which dipyrone inhibited the neurogenic algesic response induced by formalin and capsaicin was further investigated in the present study by means of both pharmacological and neurochemical procedures. The results demonstrated that the activation of the opioid naloxone-sensitive pathway is unlikely to be involved in the antinociception caused by dipyrone, as naloxone, under conditions where it fully reversed morphine-induced antinociception, had no effect against the dipyrone action. Similarly, the antinociception elicited by dipyrone also appears to be independent of an interaction with $GABA_B$ receptors and the serotonin system. These views are based in the fact that phaclofen, a selective $GABA_B$ receptor antagonist, and DL-*p*-chlorophenylalanine methyl ester hydrochloride, an inhibitor of serotonin synthesis, significantly reversed baclofen (Malcangio et al., 1991; Vaz et al., 1996)-and morphine-induced antinociception, respectively (Taber and Lantranyi, 1981; Von Voigtlander et al., 1984; Vaz et al., 1996). However, as assessed in the formalin model of pain, treatment of the animals with phaclofen or with DL-*p*-chlorophenylalanine methyl ester hydrochloride failed to interfere with dipyrone-induced antinociception. The activation of ATP-sensitive K^+ channels also does not appear to have an important role in dipyrone-induced antinociception in the formalin test, as treatment of the animals with glibenclamide, under conditions where the antinociception caused by morphine was markedly reversed (Raffa and Martinez, 1995; Shewade

and Ramaswamy, 1995), had no effect on dipyrone's antinociceptive action when tested against either phases of the formalin test.

It has been reported that dipyrone reverses, in a dose-related manner, the hyperalgesia caused by subplantar injection of carrageenan and prostaglandin E_2 into the rat paw, by a mechanism which seems to be largely modulated by the nitric oxide pathway (Ferreira, 1993; Tonussi and Ferreira, 1994; Lorenzetti and Ferreira, 1996). Our results contrast somewhat with these reports for the inflammatory model of hyperalgesia, and show that the antinociception caused by dipyrone against formalin-induced pain is unlikely to involve an interaction with nitric oxide or a nitric oxide-related substance because: 1) treatment of the animals with L-arginine, a precursor of nitric oxide, under conditions where it consistently reversed the antinociception caused by N^G-nitro-L-arginine (a nitric oxide synthase inhibitor) (Santos et al., 1995; Vaz et al., 1996), failed to interfere with dipyrone-induced antinociception, and 2) dipyrone, even at very high concentrations 'in vitro', had no significant effect against the synthesis of nitric oxide in rat brains. Another discrepancy between our findings and those recently reported by Lorenzetti and Ferreira (1996) is the absence of a central analgesic effect of dipyrone when it was tested in prostaglandin-induced hyperalgesia in the rat paw. A central analgesic action for dipyrone has also been suggested by Carlsson et al. (1986) and Carlsson and Jurna (1987) when this drug was assessed in the tail-flick test. This suggests that, depending on the model of nociception and/or animal used, dipyrone may present with different mechanisms of analgesic action.

It is now widely accepted on the basis of behavioural and electrophysiological studies that amino acid excitatory receptors are implicated in modulating thermal hyperalgesia and pain following nerve injury. Thus, selective antagonists of these receptors reveal a potent analgesic action, although some also cause motor dysfunction of the animals (Mao et al., 1992; Eisenberg et al., 1993; Coderre and Van Empel, 1994; Ferreira and Lorenzetti, 1994). The results of the present study show for the first time that dipyrone antinociception could involve, at least in part, its ability to interact with excitatory amino acids, as demonstrated by the fact that dipyrone caused dose-dependent, systemic, spinal and supraspinal inhibition of the hyperalgesia induced by i.t. injection of glutamate in mice. It is interesting to mention that dipyrone, except when it was given i.c.v., was about 14 to 45-fold more potent to reverse the hyperalgesic effect caused by i.t. injection of glutamate, than for its antinociceptive action observed in formalin- and capsaicin-induced pain. Such results strongly suggest a preferential effect of dipyrone on excitatory amino acid hyperalgesic actions. Recently, Liu et al. (1997) reported that i.t. injection of NMDA caused pain and release of substance P from small-diameter primary sensitive fibres. These NMDA effects are also associated with internalisation of substance P receptors into the dorsal horn neurones and changes in their structures (Liu et al., 1997). All the effects caused by i.t. NMDA injection were prevented by selective NMDA antagonists or NK_1 receptor antagonists, as well as by neonatal capsaicin treatment. These and other data reported in the literature (Dougherty and Willis, 1991; Rusin et al., 1993) show a complex interaction between excitatory amino acids and the tachykinin system.

Therefore, the possibility of a modulatory action of dipyrone on excitatory amino acid-mediating substance P release and/or action cannot be discarded and this matter requires further investigation. Finally, we investigated whether dipyrone antinociception could be associated with direct inhibition of [^{3}H]glutamate binding sites in both rat and mouse cerebral cortical membranes. Despite the concentration-dependent systemic, spinal and supraspinal anti-hyperalgesic action of dipyrone when assessed with glutamate-induced hyperalgesia, this drug, at higher concentrations, inhibited partially (about 37%) the glutamate binding sites in rat and mouse cortical membranes. Such results suggest that dipyrone's analgesic action is unlikely to be associated exclusively with direct inhibition of glutamate binding sites, and is probably associated with the modulation of both pre- and postsynaptic glutamate release and/or action.

With regard to the transduction mechanism involved in dipyrone antinociception, the results of the present study show for the first time that this drug, in contrast to morphine, at the analgesic range of doses, does not interact with G_i protein sensitive to treatment with pertussis toxin. Thus, the i.c.v. treatment of animals with pertussis toxin, at a dose that had been demonstrated to suppress the analgesic effect caused by morphine through ADP ribosylation (Sánchez-Blázquez and Garzón, 1991; Hernandez et al., 1995), had no significant effect on dipyrone's antinociception when assessed in the formalin test. Such results strongly indicate that dipyrone analgesia, in contrast with morphine, is not coupled to G_o/G_i pertussis-toxin-sensitive mechanisms. Finally, the dipyrone-induced antinociception is unlikely to be modulated by adrenal gland hormones, as indicated by the fact that the same antinociceptive action was observed in adrenalectomised animals and sham-operated animals.

In summary, we have reported that dipyrone elicited a spinal and supraspinal dose-related antinociception when assessed against both formalin- and capsaicin-induced neurogenic pain as well as in glutamate-induced hyperalgesia in the mouse. The precise mechanism underlying the antinociceptive action of dipyrone has yet to be determined, but it is unlikely to be associated with an interaction with opioid peptides, $GABA_B$ receptors, serotonin system, interaction with ATP-sensitive potassium channels, L-arginine nitric oxide pathway, participation of G_i/G_o protein sensitive to pertussis toxin (give by i.c.v.) or modulation by adrenal hormones. However, dipyrone's antinociception does seem to be related to its ability to modulate the excitatory amino acid release and/or action

at the spinal cord. Finally, a direct interaction of dipyrone with the binding of glutamate on its receptors might also account, at least in part, for its antinociceptive action.

Acknowledgements

This study was supported by grants from Financiadora de Estudos e Projetos (FINEP) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. A. Beirith and A.R.S. Santos are MsC and PhD students in Pharmacology, respectively, and they thank CNPq and CAPES for fellowship support.

References

Aanonsen, L.M., Wilcox, G.L., 1987. Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and sigma agonists. J. Pharmacol. Exp. Ther. 243, 9–19.

Abbate, R., Gori, A.M., Pinto, S., Attanasio, M., Paniccia, R., Coppo, M., Castellani, S., Giusti, B., Boddi, M., Neri, S.G.G., 1990. Cycloxygenase and lipoxygenase metabolite synthesis by polymorphonuclear neutrophils: in vitro effect of dipyrone. Prostaglandins Leukotrienes Essent. Fatty Acids 41, 89–93.

Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA 87, 682–685.

Carlsson, K.H., Jurna, I., 1987. The role of descending inhibition in the antinociceptive effects of the pyrazolone derivatives, metamizole (dipyrone) and aminophenazone ('Pyramidon'). Naunyn-Schmiedebergs's Arch. Pharmacol. 335, 149–154.

Carlsson, K.H., Helmreich, J., Jurna, I., 1986. Activation of inhibition from the periaqueductal grey matter mediates central analgesic effect of metamizole (dipyrone). Pain 27, 373–390.

Coderre, T.J., Melzack, R., 1992. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J. Neurosci. 12, 3665–3670.

Coderre, T.J., Van Empel, I., 1994. The utility of excitatory amino acid (EAA) antagonists as analgesic agents: II. Assessment of the antinociceptive activity of combinations of competitive and non-competitive NMDA antagonists with agents acting at allosteric glycine and polyamine receptor sites. Pain 59, 353–359.

Corrêa, C.R., Calixto, J.B., 1993. Evidence for participation of B1 and B2 kinin receptors in formalin-induced nociceptive response in the mouse. Br. J. Pharmacol. 110, 193–198.

Corrêa, C.R., Kyle, D.J., Chakravarty, S., Calixto, J.B., 1996. Antinociceptive profile of the pseudopeptide B2 bradykinin receptor antagonist NPC 18688 in mice. Br. J. Pharmacol. 117, 552–558.

D'Amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72, 74–79.

Dickenson, A.H., 1995. Central acute pain mechanisms. Ann. Med. 27, 223–227.

Dray, A., 1995. Inflammatory mediators of pain. Br. J. Anaesth. 75, 125–131.

Dougherty, P.M., Willis, W.D., 1991. Enhancement of spinothalamic neuron responses to chemical and mechanical stimuli following combined micro-iontophoretic application of *N*-methyl-D-aspartic acid and substance P. Pain 47, 85–93.

Duarte, I.D., Santos, I.R., Lorenzetti, B.B., Ferreira, S.H., 1992. Analgesia by direct antagonism of nociceptor sensitization involves the arginine-nitric oxide-cGMP pathway. Eur. J. Pharmacol. 217, 225–227.

Eddy, N.B., Leimbach, D., 1953. Synthetic analgesics: II. Dithienylbutenyl and dithienylbutylamines. J. Pharmacol. Exp. Ther. 107, 385–393.

Eisenberg, E., Vos, B.P., Strassman, A.M., 1993. The NMDA antagonist memantine blocks pain behavior in a rat model of formalin-induced facial pain. Pain 54, 301–307.

Engelhardt, G., Homma, D., Schlegel, K., Utzmann, R., Schnitzler, C., 1995. Antiinflammatory, analgesic, antipyretic and related properties of meloxicam, a new non-steroidal anti-inflammatory agent with favourable gastrointestinal tolerance. Inflamm. Res. 44, 423–433.

Ferreira, S.H., 1993. The role of interleukinin and nitric oxide in the mediation of inflammatory pain and its control by peripheral analgesics. Drugs 46, 1–9.

Ferreira, S.H., Lorenzetti, B.B., 1994. Glutamate spinal retrograde sensitization of primary sensory neurons associated with nociception. Neuropharmacology 33, 1479–1485.

Ferreira, S.H., Lorenzetti, B.B., Correa, F.M.A., 1978. Central and peripheral antialgic action of aspirin-like drugs. Eur. J. Pharmacol. 53, 39–48.

Gelgor, L., Cartmell, S., Mitchell, D., 1992. Intracerebroventricular micro-injections of non-steroidal anti-inflammatory drug abolish reperfusion hyperalgesia in the rat's tail. Pain 50, 323–329.

Granados, S.V., Flores, M.F.J., Castaneda, H.G., Lopez, M.F.J., 1995. Evidence for the involvement of nitric oxide in the antinociceptive effect of ketorolac. Eur. J. Pharmacol. 277, 281–284.

Haley, J.E., Sullivan, A.F., Dickenson, A.H., 1990. Evidence for spinal *N*-methyl-D-aspartate receptor involvement prolonged chemical nociception in the rat. Brain Res. 518, 218–226.

Hernandez, A., Soto-Moyano, R., Mestre, C., Eschalier, A., Pelissier, T., Paeile, C., Contreras, E., 1995. Intrathecal pertussis toxin but not cyclic AMP blocks kappa opioid-induced antinociception in rat. Int. J. Neurosci. 81, 193–197.

Hylden, J.K.L., Wilcox, G.L., 1980. Intrathecal morphine in mice: A new technique. Eur. J. Pharmacol. 67, 313–316.

Knowles, R.G., Palacios, M., Palmer, R.M.J., Moncada, S., 1990. Kinetic characteristics of nitric synthase from rat brain. Biochem. J. 269, 207–210.

Laursen, S.E., Belknap, J.K., 1986. Intracerebroventricular injections in mice: Some methodological refirements. J. Pharmacol. Methods 16, 355–357.

Liu, H., Mantyh, P.W., Basbaum, A.I., 1997. NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 386, 721–724.

López-Muñoz, F.J., Villalón, C.M., Terrón, J.A., Salazar, L.A., 1994. Analgesic interactions produced by dipyrone and either morphine or D-propoxyphene in the pain-induced functional impairment model in rat. Drug Dev. Res. 32, 50–57.

Lorenzetti, B.B., Ferreira, S.H., 1985. Mode of analgesic action of dipyrone: direct antagonism of inflammatory hyperalgesia. Eur. J. Pharmacol. 114, 375–381.

Lorenzetti, B.B., Ferreira, S.H., 1996. Activation of the arginine-nitric oxide pathway in primary sensory neurones contributes to dipyrone-induced spinal and peripheral analgesia. Inflamm. Res. 45, 308–311.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Malcangio, M., Ghelardini, C., Giotti, A., Malmberg-Aiello, P., Bartolini, A., 1991. CGP 35348, a new $GABA_B$ antagonist, prevents antinociception and muscle-relaxant effect by baclofen. Br. J. Pharmacol. 103, 1303–1308.

Malmberg, A.B., Yaksh, T.L., 1992. Antinociceptive actions of nonsteroidal anti-inflammatory agents on the formalin test in the rat. J. Pharmacol. Exp. Ther. 263, 136–146.

Mao, J., Price, D.D., Hayes, R.L., Lu, J., Mayer, D.J., 1992. Differential roles of NMDA and non-NMDA receptor activation in induction and maintenance of thermal hyperalgesia in rats with painful peripheral mononeuropathy. Brain Res. 598, 271–278.

Meller, S.T., Gebhart, G.F., 1993. Nitric oxide (NO) and nociceptive processing in the spinal cord. Pain 52, 127–136.

Raffa, R.B., Martinez, R.P., 1995. The 'glibenclamide-shift' of centrally-acting antinociceptive agents in mice. Brain Res. 677, 277–282.

Rosland, J.H., Hunskaar, S., Hole, K., 1990. Diazepam attenuates morphine antinociception test-dependently in mice. Pharmacol. Toxicol. 66, 382–386.

Rusin, K.I., Bleakman, D., Chard, P.S., Randic, M., Miller, R.J., 1993. Tachykinins potentiate *N*-methyl-D-aspartate responses in acutely isolated neurons from the dorsal horn. J. Neurochem. 60, 952–960.

Sánchez-Blázquez, P., Garzón, J., 1991. Cholera toxin and pertussis toxin an opioid- and -α2-mediated supraspinal analgesia in mice. Life Sci. 48, 1721–1727.

Santos, A.R.S., Calixto, J.B., 1997. Further evidence for the involvement of tachykinin receptor subtypes in formalin and capsaicin models of pain in mice. Neuropeptides 31, 381–389.

Santos, A.R.S., Cechinel Filho, V., Yunes, R.A., Calixto, J.B., 1995. Analysis of the mechanisms underlying the antinociceptive effect of the extracts of plants from the genus Phyllanthus. Gen. Pharmacol. 26, 1499–1506.

Shewade, D.G., Ramaswamy, S., 1995. Prolactin-induced analgesia is dependent on ATP-sensitive potassium channels. Clin. Exp. Pharmacol. Physiol. 22, 635–636.

Shibata, M., Ohkubo, T., Takahashi, H., Inoki, R., 1989. Modified formalin test: characteristic biphasic pain response. Pain 38, 347–352.

Shimada, S.G., Otterness, I.G., Stitt, J.T., 1994. A study of the mechanism of action of the mild analgesic dipyrone. Agents Actions 41, 188–192.

Souza, D.O., Ramírez, G., 1991. Effects of guanine nucleotides on kainic acid binding and on adenylate cyclase in chick optic tectum and cerebellum. J. Mol. Neurosci. 3, 39–45.

Taber, R.I., Lantranyi, A.V., 1981. Antagonism of the analgesic effect of opioid and non-opioid agents by *p*-chlorophenylalanine (PCPA). Eur. J. Pharmacol. 75, 215–222.

Tonussi, C.R., Ferreira, S.H., 1994. Mechanism of diclofenac analgesia: direct blockade of inflammatory sensitization. Eur. J. Pharmacol. 251, 173–179.

Tracey, D.J., Walker, J.S., 1995. Pain due to nerve damage: Are inflammatory mediators involved?. Inflamm. Res. 44, 407–411.

Vaz, Z.R., Cechinel Filho, V., Yunes, R.A., Calixto, J.B., 1996. Antinociceptive action of 2-(4-bromobenzoyl)-3-methyl-4,6-dimethoxy benzofuran, a novel Xanthoxyline derivative on chemical and thermal models of nociception in mice. J. Phamacol. Exp. Ther. 278, 304–312.

Von Voigtlander, P.F., Lewis, R.A., Neff, G.L., 1984. Kappa-opioid analgesia is dependent on serotonergic mechanisms. J. Pharmacol. Exp. Ther. 231, 270–274.

Wolff, D.J., Datto, G.A., 1992. Identification and characterization of a calmodulin-dependent nitric oxide synthase from GH_3 pituitary cells. Biochem. J. 285, 201–205.

Yamamoto, T., Yaksh, T.L., 1992a. Comparison of the antinociceptive effects of pre- and post-treatment with intrathecal morphine and MK-801, a NMDA antagonist, on the formalin test in the rat. Anesthesiology 77, 757–763.

Yamamoto, T., Yaksh, T.L., 1992b. Spinal pharmacology of thermal hyperethesia induced by constriction injury of sciatic nerve: II. Excitatory amino acid antagonists. Pain 49, 121–128.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.