Role of Na\(^+\),K\(^+\)-ATPase in Morphine-Induced Antinociception

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ABSTRACT

We evaluated the modulation by Na\(^+\),K\(^+\)-ATPase inhibitors of morphine-induced antinociception in the tail-flick test and \[^{[3H]}\]naloxone binding to forebrain membranes. The antinociception induced by morphine (1–32 mg/kg, s.c.) in mice was dose-dependently antagonized by ouabain (1–10 ng/mouse, i.c.v.), which produced a significant shift to the right of the morphine dose-response curve. The i.c.v. administration of three Na\(^+\),K\(^+\)-ATPase inhibitors (ouabain at 0.1–100, digoxin at 1–1,000, and digitoxin at 10–10,000 ng/mouse) dose-dependently antagonized the antinociceptive effect of morphine (4 mg/kg, s.c.) in mice, with the following order of potency: ouabain > digoxin > digitoxin. This effect cannot be explained by any interaction at opioid receptors, since none of these Na\(^+\),K\(^+\)-ATPase inhibitors displaced \[^{[3H]}\]naloxone from its binding sites, whereas naloxone did so in a concentration-dependent manner. The antinociception induced by morphine (5 mg/kg, s.c.) in rats was antagonized by the i.c.v. administration of ouabain at 10 ng/rat, whereas it was not significantly modified by intrathecally administered ouabain (10 and 100 ng/rat). These results suggest that the activation of Na\(^+\),K\(^+\)-ATPase plays a role in the supraspinal, but not spinal, antinociceptive effect of morphine.

Several authors, including us, have reported an activation of neuronal Na\(^+\),K\(^+\)-ATPase activity by morphine and endomorphin-1 in vitro (Hajek et al., 1985; Nishikawa et al., 1990; Masocha et al., 2002; Horvath et al., 2003) and by morphine in vivo (Desaiiah and Ho, 1977; Sharma et al., 1998). Furthermore, it has been demonstrated that electroacupuncture produces antinociception and a stimulation of Na\(^+\),K\(^+\)-ATPase activity that is blocked by i.p. injection of naloxone prior to electroacupuncture (Lee and Sun, 1984). These results suggest a link between activation of Na\(^+\),K\(^+\)-ATPase and opioid-induced antinociception. Na\(^+\),K\(^+\)-ATPase represents the pharmacological receptor of digitalis and strophanthus glycosides, which specifically block the activity of this enzyme (Wallick et al., 1979; Lingrel et al., 1997, 1998). Therefore, if opioid-receptor agonists stimulate Na\(^+\),K\(^+\)-ATPase activity and this action plays a role in their antinociceptive effects, Na\(^+\),K\(^+\)-ATPase inhibitors (e.g., ouabain, digoxin, and digitoxin) might be able to antagonize such antinociceptive effects. This hypothesis has been little tested, and discrepant results have been reported. It has been shown that i.t. administration of ouabain enhanced (Zeng et al., 1999) or did not modify the antinociception induced by i.t. morphine (Horvath et al., 2003) and produced a small antagonism of the effect of low (but not high) doses of i.t. endomorphin-1 (Horvath et al., 2003). The antinociception induced by the i.t. administration of \(\mu\)-opioid agonists is mainly due to the activation of nalofoxazine-insensitive (\(\mu_2\)) receptors (Paul et al., 1989; Fasternak and Letchworth, 1999), whereas the activation of Na\(^+\),K\(^+\)-ATPase activity by morphine is nalofoxazine-sensitive (\(\mu_1\)-mediated) (Masocha et al., 2002). This prompted us to evaluate the effect of i.c.v. administration of several Na\(^+\),K\(^+\)-ATPase inhibitors (ouabain, digoxin, and digitoxin) on the antinociception induced by the s.c. administration of morphine, which produced a nalofoxazine-sensitive antinociception (Pick et al., 1991). Since we found a clear antagonism of the antinociceptive effect of morphine in mice by i.c.v.-administered digitalis glycosides, whereas all of the above-mentioned studies that evaluate the interaction of ouabain-opioid agonists were performed in rats with i.t. administration, we compared the effect of i.c.v. and i.t. ouabain on the antinociception induced by s.c. morphine in rats. Moreover, to discard the possibility that the effect of digitalis glycosides on morphine-induced antinociception was due to a direct interaction with the opioid receptors, we evaluated the effect of these drugs on \[^{[3H]}\]naloxone binding, a marker of \(\mu\)-, \(\delta\)-, and \(\kappa\)-opioid receptors (Satoh and Minami, 1995), to mice forebrain membranes.

ABBREVIATIONS: AUC, area under the curve; ANOVA, analysis of variance.
Materials and Methods

Animals. The experiments were performed in CD-1 Swiss mice (Charles River Laboratories España S.A., Barcelona, Spain) weighing 25 to 30 g and Wistar rats (animal farm of University of Szeged, Hungary) weighing 250 to 350 g. The animals were housed in temperature-controlled (22 ± 1°C) rooms with a 12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM) and free access to food and water. All experiments were performed during the same period of the day (8:00 AM to 4:00 PM) to exclude diurnal variations in pharmacological effects. The animals were randomly assigned to treatment groups (n = 6–15/group), and the observer was blind to the treatment administered.

The animals were handled in compliance with European Communities Council Directive 86/609 for the care of laboratory animals and ethical guidelines for research in experimental pain with conscious animals (Zimmermann, 1983). All procedures were approved by the animal care committees.

Drugs. The drugs used in the studies performed in mice and their providers were: morphine HCl (General Directorate of Pharmacy and Drugs, Spanish Ministry of Health, Madrid, Spain), naloxone, ouabain, digoxin, digitoxin (all from Sigma-Aldrich Quimica SA, Madrid, Spain), and [3H]naloxone (PerkinElmer Life Sciences, Boston, MA). The drugs used in the studies performed in rats and their providers were: morphine HCl (ICN alkaloida Chemical Co., Ltd., Budapest, Hungary), ouabain (Sigma-Aldrich Kft, Budapest, Hungary), ketamine HCl (a generous gift from Renate Schwarz, Pfizer Med-Inform, Vienna, Austria), and xylazine HCl (Bayer, Leverkusen, Germany).

Preparation of Mice Forebrain P2 Membranes. Mice forebrain crude synaptosomal pellets were isolated as previously described in detail (Gonzalez et al., 2001). Briefly, mice forebrains were immersed in tubes containing ice-cold isolation medium 1 (320 mM sucrose, 3 mM EDTA tetrasodium salt, and 10 mM HEPES, pH 7.4) and were homogenized with a Polytron homogenizer (model PT10-35; Kinematica AG, Basel, Switzerland). The homogenates were centrifuged (Avanti 30; Beckman Coulter España, S. A., Madrid, Spain) at 1,000g for 10 min at 4°C; the resulting pellets were discarded, and the supernatants were centrifuged again under the same conditions. The final supernatant was then centrifuged at 17,000g for 20 min to obtain the crude synaptosomal pellet (P2 pellet). Then the pellet was dissolved in the appropriate incubation medium for binding experiments (as described under Binding Experiments), and the protein concentration was determined by a modified version of the Lowry et al. (1951) method.

Binding Experiments. The P2 pellet, obtained as described above, was dissolved in 50 mM Tris, pH 7.4. Naloxone was dissolved in 50 mM Tris, pH 7.4, whereas ouabain, digoxin, and digitoxin were dissolved in absolute ethanol to make up a 1-mM solution from which further dilutions were made with buffer (50 mM Tris, pH 7.4). For measuring total binding, we incubated in triplicate 20 μl of unlabeled drug (0.1 nM-10 μM) or its solvent, 460 μl of P2 membrane fraction (0.6 mg/ml), and 20 μl of [3H]naloxone (2 nM) at 30°C during 30 min as previously described (Freissmuth et al., 1993). For measuring nonspecific binding, 10 μM of unlabeled naloxone was added to the medium. At the end of the incubation period, the reaction was stopped by adding 5 ml of 50 mM Tris, pH 7.4 at 4°C. Each membrane solution was immediately filtered under vacuum through Whatman GF/B glass fiber filters (SEMAT Technical Ltd., St. Albans, Hertfordshire, UK; previously humidified with Tris) with a Brandel cell harvester (model M-12T; SEMAT Technical Ltd.) and washed twice with 5 ml of 50 mM Tris HCl, pH 7.4, at 4°C. The filters were transferred to scintillation counting vials to which 4 ml of liquid scintillation cocktail (Optiphase Hisafe 2; PerkinElmer Wallac, Loughborough, Leicestershire, UK) was added and left to equilibrate in the dark for 12 h. The radioactivity retained on the filter was measured using a liquid scintillation spectrometer (Beckman Coulter, Inc.) with an efficiency of 52%. Specific binding was calculated by subtracting nonspecific binding from total binding.

Drug Treatments and Assessment of Antinociception in Mice. Morphine was dissolved in ultrapure water (puruer than type 1 in the National Committee for Clinical Laboratory Standards/College of American Pathologists water quality standards) and injected s.c. in a volume of 5 ml/kg. The Na+,-K+-ATPase inhibitors ouabain, digoxin, and digitoxin were dissolved in 1% Tween 80 in ultrapure water and injected i.c.v. in a volume of 5 μl/mouse. The control animals received the same volume of vehicles. The s.c. injections were done in the interscapular region. The i.c.v. injections were done in the right lateral cerebral ventricle of mice according to the method described previously (Ocaña et al., 1993). Briefly, the injection site was identified according to the method by Haley and McCormick (1957), and the drug solution was injected with a 10-μl Hamilton syringe with a sleeve around the needle to prevent the latter from penetrating more than 3 mm into the skull. After the experiments were done, the position of the injection was evaluated in each brain, and the results from animals in which the tip of the needle did not reach the lateral ventricle were discarded.

The antinociceptive effect of the treatments was evaluated using a tail-flick test as previously described (Ocaña et al., 1993). Briefly, the animals were restrained in a Plexiglas tube and placed on the tail-flick apparatus (LI 7100; Leta SA, Barcelona, Spain). A noxious beam of light was focused on the tail about 4 cm from the tip, and the latency to tail-flick was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 3 and 5 s; this intensity was never changed, and any animal whose baseline latency was outside the pre-established limits was excluded from the experiments. Two baseline tail-flick latencies were recorded within 20 min before all injections. At time 0, the animals received an i.v. injection of ouabain, digoxin, digitoxin, or their solvent and immediately thereafter an s.c. injection of morphine or its solvent. The end of the last injection was considered as time 0; from this time, tail-flick latencies were measured again at 10, 20, 30, 45, 60, 90, and 120 min after treatment. The cutoff time was 10 s.

The area under the curve (AUC) of tail-flick latency against time was calculated for each animal using the GraphPad Prism version 3.00 for Windows (GraphPad Software Inc., San Diego, CA). The degree of antinociception was determined according to the formula: % antinociception = [(AUC0– AUCt) / (AUCmax – AUC0)] × 100, where the AUC0 and AUCt are the areas under the curve for drug- and vehicle-treated mice, respectively, and AUCmax is the area under the curve of the he curve of maximum possible antinociception (10 s in each determination).

Drug Treatments and Assessment of Antinociception in Rats. Morphine was dissolved in saline and injected s.c. in a volume of 2 ml/kg. Ouabain was dissolved in 1% Tween 80 in water and injected i.t. or i.c.v. in a volume of 5 μl/rat. Control animals received the same volume of the vehicles. For the intrathecal drug administration, the rats were surgically prepared under ketamine-xylazine anesthesia (72 and 8 mg/kg intraperitoneally, respectively). An intrathecal catheter (PE-10 tubing) was inserted through a small opening in the cisterna magna and passed 8.5 cm caudally into the intrathecal space as previously described (Yaksh and Rudy, 1976). For i.c.v. drug administration, the rats were anesthetized as described above and placed in a stereotoxic apparatus. A stainless steel 23-gauge cannula was placed into the right lateral ventricle and fixed to the skull with dental cement. Coordinates were 0.5 mm caudal and 1.5 mm lateral to the bregma, with the cannula extending 3.5 mm ventral to the skull surface. Injections were performed through a 29-gauge internal cannula, which extended 1.0 mm beyond the guide cannula tip. The internal cannula was attached to a Hamilton 10-μl syringe with a polyethylene tubing (PE-20), allowing the animals to move freely during the injection period. After surgery (both i.t. and i.c.v.), the rats were housed individually, had free access to food and water, and were allowed to recover for at least 4 days before use. Rats exhibiting postoperative neurologic deficits were not used.
Each animal was studied twice in an experimental series, with an interval of 7 to 8 days between studies. After experimental use, each rat was killed with an overdose of pentobarbital, and 1% methylene blue was injected to confirm the position of the catheter (i.t.) or cannula (i.c.v.) and probable spread of the injection. If the position was not right, the results obtained in the animal were discarded.

The antinociceptive effect of the treatments was evaluated using a tail-flick test as previously described (Horvath et al., 1990). The reaction time was determined by immersing the lower 5-cm portion of the tail in hot water (51.5°C) until a tail-withdrawal response was observed. The basal latency was 8.9 ± 0.32 s, and the cutoff time was 20 s. The tail-flick latencies were recorded immediately before and at 10, 30, 60, 90, and 120 min after the drug injections of morphine alone or associated to ouabain or its' solvent. The AUC of tail-flick latency against time and the degree of antinociception in each animal were calculated as described in the previous section but using 20 s in each determination to calculate the AUC. In the experiments where the effect of ouabain alone was tested, the drug was injected cumulatively (1, 10, 100, and 1,000 ng).

**Data Analysis.** The values of IC₅₀ (concentration of unlabeled drug that inhibited 50% of specific [³H]naloxone binding), ED₅₀ (dose of morphine that produced half of the maximal antinociception), and Eₘₐₓ (maximum antinociception produced) were calculated from the concentration-response curves or dose-response curves using nonlinear regression analysis with GraphPad Prism version 3.00. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison test or when the AUC of antinociception along time was analyzed, the area under the curve of tail-flick latency along time (as described under Materials and Methods). Each point represents the mean ± S.E.M. of the values obtained from 7 to 14 animals. Statistically significant differences in comparison with morphine + vehicle: *, p < 0.05 and **, p < 0.01 (two-way ANOVA followed by Bonferroni test).

**Results**

**Effect of i.c.v. Ouabain on the Antinociception Induced by Morphine in Mice.** The analysis of AUC values showed that the administration of morphine (1–32 mg/kg, s.c.) together with the vehicle of ouabain (i.c.v.) produced a dose-dependent increase in the percentage of antinociception in the treated mice (Fig. 1). Using nonlinear regression analysis, we fitted the data to a sigmoid curve, which made it possible to calculate Eₘₐₓ as 78.40 ± 2.94% of antinociception and ED₅₀ as 2.16 ± 0.20 mg/kg. The treatment of mice with ouabain (1 and 10 ng/mouse, i.c.v.) produced a dose-dependent antagonism of the ability of morphine to induce antinociception and displaced the dose-response curve of morphine to the right (Fig. 1). The maximum efficacy of morphine was dose-dependently reduced by ouabain (Eₘₐₓ = 67.49 ± 2.19 and 63.45 ± 1.58% for mice treated with 1 and 10 ng/mouse, respectively). The ED₅₀ of morphine was dose-dependently increased in the animals treated with ouabain (ED₅₀ = 5.02 ± 0.34 and 6.43 ± 0.34 mg/kg for mice treated with 1 and 10 ng/mouse, respectively).

The s.c. administration of morphine (4 mg/kg) together with the i.c.v. injection of the vehicle of ouabain induced an increase in tail-flick latency in a time-dependent manner (Fig. 2, left side). The maximum effect was observed at 30 min and posteriorly decayed progressively. Ouabain (0.1–10 ng/mouse, i.c.v.) dose-dependently antagonized this effect of morphine; the antagonism was particularly evident from 30 to 120 min after morphine administration (Fig. 2, left side). The administration of ouabain (0.1–100 ng/mouse, i.c.v.) together with the solvent of morphine (s.c.) did not significantly modify tail-flick latency at any of the times and doses tested (data not shown).

When the AUC of antinociception along time was analyzed, ouabain (0.1–10 ng/mouse, i.c.v.) antagonized the antinociceptive effect of morphine (4 mg/kg, s.c.) in a dose-dependent manner (Fig. 2, right side). The maximum antagonism by ouabain was produced at 10 ng/mouse, which reduced the percent antinociception from 69 ± 7.4% (morphine + vehicle) to 20 ± 8.9%. A higher dose of ouabain (100 ng/mouse, i.c.v.) produced a slightly lower antagonism of morphine antinociception (Fig. 2, right side).

**Effects of Several Na⁺,K⁺-ATPase Inhibitors on the Antinociception Induced by Morphine in Mice.** The s.c. administration of morphine (4 mg/kg) together with the vehicle of the Na⁺,K⁺-ATPase inhibitors (i.c.v.) produced a percentage of antinociception of 74.77 ± 6.75% (Fig. 3). The antinociception induced by morphine (4 mg/kg, s.c.) was dose-dependently antagonized by the i.c.v administration of the three Na⁺,K⁺-ATPase inhibitors (ouabain at 0.1 to 100, digoxin at 1 to 1,000, and digitoxin at 10 to 10,000 ng/mouse), with the following order of potency: ouabain > digoxin > digitoxin. As seen in Fig. 3, the Na⁺,K⁺-ATPase inhibitors did not antagonize completely the antinociception produced by morphine (4 mg/kg, s.c.). The least percentage of antinociception produced by morphine (4 mg/kg, s.c.) associated with the highest dose of the Na⁺,K⁺-ATPase inhibitor used was 28.61 ± 7.08% for ouabain (100 ng/mouse, i.c.v.), 37.08 ± 5.7% for digoxin (1,000 ng/mouse, i.c.v.), and 28.97 ± 9.46% for digitoxin (10,000 ng/mouse, i.c.v.). Nonstatistically significant differences were found between these values of antinociception. Higher doses of Na⁺,K⁺-ATPase inhibitors were not tested, as they caused hyperexcitability and convulsions in some animals.
Effects of Na\(^{+}\),K\(^{+}\)-ATPase Inhibitors and Naloxone on \[^{3}H\]Naloxone Binding to Mice Forebrain Membranes. Fig. 4 shows the competition for \[^{3}H\]naloxone binding sites by naloxone and the Na\(^{+}\),K\(^{+}\)-ATPase inhibitors (ouabain, digoxin, and digitoxin). Naloxone (0.1 nM-10 \(\mu\)M) concentration-dependently displaced \[^{3}H\]naloxone from its binding sites to forebrain membranes, with an IC\(_{50}\) of 3.58 \(\pm\) 0.18 nM. In contrast, ouabain, digoxin, and digitoxin, even at concentrations of 10 \(\mu\)M, did not significantly modify \[^{3}H\]naloxone-specific binding.

Effect of i.c.v. Administration of Ouabain on the Antinociception Induced by Morphine in Rats. The s.c. administration of morphine (5 mg/kg) together with the i.c.v. injection of the vehicle of ouabain induced an increase in tail-flick latency in a time-dependent manner. The maximum effect was observed at 30 min and posteriorly decayed slowly (Fig. 5, left side). Ouabain (10 ng/rat, i.c.v.) antagonized this effect of morphine; the antagonism was evident from 30 to 120 min after morphine administration, although only reaching statistical significance at 90 and 120 min. On the other hand, a higher dose of ouabain (100 ng/rat, i.c.v.) did not significantly antagonize morphine-induced antinociception at any time (Fig. 5, left side). The administration of ouabain (10 and 100 ng/rat, i.c.v.) together with the solvent for morphine (s.c.) did not modify the tail-flick latency at any of the doses or times tested (data not shown). When the area under the curve of antinociception along time was analyzed, ouabain (10 ng/rat, i.c.v.) significantly antagonized the antinociceptive effect of morphine (5 mg/kg, s.c.), whereas the highest dose of ouabain used (100 ng/rat, i.c.v.) did not (Fig. 5, right side).

Effect of i.t. Administration of Ouabain on the Antinociception Induced by Morphine in Rats. The administration of morphine (5 mg/kg, s.c.) together with the vehicle

Fig. 2. Antagonism by i.c.v. treatment with different doses of ouabain of the antinociception induced by morphine (4 mg/kg, s.c.) in a tail-flick test in mice. The percentage of antinociception was calculated from the area under the curve of tail-flick latency along time (as described under Materials and Methods). Each column represents the mean \(\pm\) S.E.M of the values obtained from 10 to 12 animals. Statistically significant differences in comparison with morphine + vehicle: *, \(p < 0.05\) and **, \(p < 0.01\) (one-way ANOVA followed by Newman-Keuls test).

Fig. 3. Antagonism by i.c.v. treatment with different doses of ouabain, digoxin, and digitoxin of the antinociception induced by morphine (4 mg/kg, s.c.) in a tail-flick test in mice. The percentage of antinociception was calculated from the area under the curve of tail-flick latency along time (as described under Materials and Methods). The shaded area represents the mean \(\pm\) S.E.M of the antinociception produced in the animals treated with morphine + vehicle. Each point represents the mean of the values obtained from 6 to 12 animals. Statistically significant differences in comparison with morphine + vehicle: *, \(p < 0.05\) and **, \(p < 0.01\) (one-way ANOVA followed by Newman-Keuls test).
of ouabain induced an increase in tail-flick latency that reached a maximum at 30 min and decreased at 120 min (Fig. 6, left side). Ouabain (10 ng/rat, i.t.) significantly antagonized this effect of morphine only at 60 min after drug administration. On the other hand, a higher dose of ouabain (100 ng/rat, i.t.) had a significant synergistic effect with morphine (5 mg/kg, s.c.) only at 10 min after drug administration (Fig. 6, left side). When the area under the curve of antinociception along time was analyzed, none of the doses of ouabain (10 and 100 ng/rat, i.t.) significantly modified the antinociceptive effect of morphine (5 mg/kg, s.c.) at 10 min after drug administration (Fig. 6, right side). The administration of ouabain (10 and 100 ng/rat, i.t.) together with the solvent of morphine (s.c.) did not modify tail-flick latency at any of the doses tested (data not shown).

**Discussion**

The present study found two main results: 1) that ouabain, digoxin, and digitoxin did not have affinity for opioid receptors but when i.c.v. administered antagonized the antinociceptive effect of s.c. morphine in mice; and 2) that ouabain administered i.c.v., but not i.t., antagonized the antinociception induced by s.c. morphine in rats.

The i.c.v. administration of several digitalis glycosides dose-dependently antagonized the antinociceptive effect of morphine in mice. Under the same experimental conditions, these drugs, when i.c.v. injected (ouabain at 10 and 100, digoxin at 100 and 1000, and digitoxin at 1000 and 10000 ng/mouse), did not modify the antinociceptive effect of the κ-opioid receptor agonist trans-(d,l)-3,4-dichloro-N,N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetonamide methanesulfonate (8 mg/kg, s.c.) (manuscript in preparation). This indicates that the antagonism by digitalis glycosides of morphine-induced antinociception is specific and not an indiscriminate antagonism of the antinociception induced by any drug.

The antagonism of the antinociceptive effect of morphine by digitalis glycosides cannot be explained by a direct interaction of these drugs with the opioid receptors, since none of these drugs significantly displaced [3H]naloxone, a marker of μ-, δ-, and κ-opioid receptors (Sato and Minami, 1995), from its binding sites to mice forebrain membranes. On the other hand, morphine increases Na+,K+-ATPase activity (Nishikawa et al., 1990; Sharma et al., 1998; Masocha et al., 2002), and digitalis glycosides specifically block the activity of this enzyme (Wallick et al., 1979; Lingrel et al., 1997, 1998); therefore, the ability of digitalis glycosides to antagonize morphine-induced antinociception can be due to their ability to block the Na+,K+-ATPase activity. In support of this hypothesis, it is interesting to note that the order of potency of the digitalis glycosides in antagonizing morphine-induced antinociception (ouabain > digoxin > digitoxin) is the same as their order of potency in inhibiting [3H]ouabain binding in brain membranes (Acuña-Castroviejo et al., 1992). The fact that about 30% of the antinociception induced by morphine in mice was insensitive to digitalis glycosides might be due to the fact that beside the stimulation of Na+,K+-ATPase activity, other mechanisms, like the opening of KATP channels (Ocaña et al., 1993, 1995; Raffa and Martinez, 1995), the closing of voltage-dependent Ca2+- channels (Del Pozo et al., 1990; Dierssen et al., 1990), or the inhibition of adenylyl cyclase (Suh et al., 1995, 1996), are involved in the antinociceptive effect of morphine. Another possible reason to explain the partial antagonism by digitalis glycosides of morphine-induced antinociception might be related to the different routes of administration of both drugs. When morphine is administered peripherally (s.c. or i.p.), it is distributed in supraspinal and spinal sites (Miyamoto et al., 1991), and the activation of opioid receptors located in both sites is essential for the production of its antinociceptive effect (Yeung and Rudy, 1980a,b; Roerig and Fujimoto, 1989). When digitalis glycosides are i.c.v. administered, they are expected to be mainly distributed in the supraspinal space. Then, the portion of morphine-induced antinociception not blocked by i.c.v. Na+,K+-ATPase inhibitors could have been, at least partially, spinally mediated. In support of this notion, we found that i.t. administration of ouabain did not antagonize the antinociception induced by i.t. morphine in rats (Horvath et al., 2003) nor that produced by s.c. morphine in rats (present study).

In agreement with the data obtained in mice, we observed that the i.c.v. administration of ouabain (10 ng/rat) reduced the antinociception induced by morphine in rats. However, the antagonism of morphine-induced antinociception produced by i.c.v. administration of ouabain was less in rats than in mice. This might be attributed to the species-specific differences of the Na+,K+-ATPase α-subunit (Feschenko et al., 1997) and variations between species in residues that convey ouabain sensitivity within the first and second transmembrane helices hairpin of the Na+,K+-ATPase (Lingrel et al., 1997). It could also be speculated that, in the lower degree of antagonism by i.c.v. ouabain in rats compared with mice, a greater relative contribution of spinal versus supraspinal sites of action for s.c. morphine-induced antinociception in rats than mice might have played a role. On the other hand, it is interesting to note that a higher dose of ouabain (100 ng/rat) produces less antagonism of morphine effect than 10 ng/rat. A similar fact was observed in mice (see Fig. 2, right side), which suggests that a relatively
A high dose of ouabain may produce effects additional to those of low doses that can counteract the antagonism induced by the low doses of ouabain. In fact, the only study that has evaluated previously the effect of i.c.v. ouabain on morphine-induced antinociception found that 100 ng of ouabain did not antagonize the effect of morphine (Calcutt et al., 1971).

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**Fig. 5.** Antagonism by i.c.v. treatment with ouabain of antinociception induced by morphine (5 mg/kg, s.c.) in a tail-flick test in rats. Left side, time course of the tail-flick latency times for various combinations of morphine (5 mg/kg, s.c.) or its vehicle and ouabain (10 and 100 ng/rat, i.c.v.) or its vehicle. Each point represents the mean ± S.E.M of the values obtained from 6 to 10 animals. Statistically significant differences in comparison with morphine + vehicle: ••, p < 0.01 (two-way ANOVA followed by Bonferroni test). Right side, antagonism by treatment with ouabain (10 and 100 ng/rat, i.c.v.) of the antinociception induced by morphine (5 mg/kg, s.c.). The solid column represents the effect of morphine + ouabain vehicle. The percentage of antinociception was calculated from the area under the curve of tail-flick latency along time (as described under Materials and Methods). Each column represents the mean ± S.E.M of the values obtained from 6 to 10 animals. Statistically significant differences in comparison with morphine + vehicle: •, p < 0.05 (one-way ANOVA followed by Newman-Keuls test).

**Fig. 6.** Effect of i.t. treatment with ouabain on the antinociception induced by morphine (5 mg/kg, s.c.) in a tail-flick test in rats. Left side, time course of the tail-flick latency times for various combinations of morphine (5 mg/kg, s.c.) or its vehicle and ouabain (10 and 100 ng/rat, i.t.) or its vehicle. Each point represents the mean ± S.E.M of the values obtained from 7 to 10 animals. Statistically significant differences in comparison with morphine + vehicle: •, p < 0.05 and ••, p < 0.01 (two-way ANOVA followed by Bonferroni test). Right side, effect of treatment with ouabain (10 and 100 ng/rat, i.t.) on the antinociception induced by morphine (5 mg/kg, s.c.). The solid column represents the effect of morphine + ouabain vehicle. The percentage of antinociception was calculated from the area under the curve of tail-flick latency along time (as described under Materials and Methods). Each column represents the mean ± S.E.M of the values obtained from 7 to 10 animals. No statistically significant differences were observed in comparison with morphine + vehicle (one-way ANOVA).
On the other hand, 10 ng/rat ouabain i.t. reduced the increase of tail-flick latency induced by morphine only at 60 min after drug administration, whereas a higher dose of ouabain (100 ng/rat, i.t.) had a synergistic effect on the morphine-induced increase in tail-flick latency only at 10 min after drug administration. However, both doses of ouabain (10 and 100 ng/rat, i.t.) had no significant effect on the antinociception induced by morphine in rats when a global value of antinociception (AUC of tail-flick latency along time) was used. These results demonstrate a difference between the effects of i.c.v. and i.t.-administered ouabain on the morphine-induced antinociception. Previous studies have reported that i.t.-administered ouabain enhanced (Zeng et al., 1999) or did not modify the antinociception induced by i.t. morphine (Horvath et al., 2003). Therefore, it seems that ouabain antagonized the supraspinal antinociception induced by morphine, whereas it did not significantly antagonize its spinal antinociception. The supraspinal antinociceptive effects of morphine are mediated mainly through μ1 opioid receptors, whereas the spinal antinociceptive effects are mediated through μ2 opioid receptors (Paul et al., 1989; Pasternak and Letchworth, 1999). We have demonstrated that μ1 opioid receptors have an important role in the enhancement of synaptosomal Na+,K+-ATPase activity induced by morphine (Masocha et al., 2002). Thus, one would expect an antagonism of the antinociceptive effect of morphine by ouabain administered i.c.v. but not i.t.

In conclusion, our results suggest that activation of neuronal Na+,K+-ATPase plays a role in the supraspinal, but not spinal, antinociceptive effect of morphine.

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References


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