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Distinguishing subgroups among μ-opioid receptor agonists using Na^+,K^+-ATPase as an effector mechanism

Willias Masocha [a,1], Luis G. González [b,*,1], Ahmad Agil [b]

[a] Department of Pharmacology and Therapeutics, Faculty of Pharmacy, Kuwait University, P.O. Box 24923, Safat 13110, Kuwait
[b] Department of Pharmacology and Neurosciences Institute, Faculty of Medicine, University of Granada, Avenida de la Investigación 11, E-18016 Granada, Spain

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ABSTRACT

We evaluated the effects of intracerebroventricular administration of ouabain on the antinociception induced by five μ-opioid receptor agonists in a tail flick test on female CD-1 mice and the effects of these μ-opioid receptor agonists on mice forebrain synaptosomal ouabain-sensitive Na^+,K^+-ATPase activity. The subcutaneous administration of the μ-opioid receptor agonists tested produced a dose-dependent antinociceptive effect. The antinociception induced by morphine (1–32 mg/kg), levorphanol (0.4–6.4 mg/kg), and buprenorphine (0.02–0.64 mg/kg) was antagonised in a dose-dependent manner by ouabain (0.001–10 ng, i.c.v.), whilst the antinociception produced by fentanyl (0.02–0.16 mg/kg) and methadone (2–10 mg/kg) was not influenced significantly by ouabain (1–100 ng, i.c.v.). Incubation *in vitro* of forebrain synaptosomes with morphine (10^{-9}–10^{-4} M), levorphanol (10^{-10}–10^{-4} M), buprenorphine (10^{-10}–10^{-5} M), or fentanyl (10^{-10}–10^{-5} M) stimulated significantly ouabain-sensitive Na^+,K^+-ATPase activity in a concentration-dependent way. The order of efficacy (using the E_{max} as a measure of intrinsic efficacy) was: morphine ($29.83 \pm 0.56\%$) > levorphanol ($18.61 \pm 1.26\%$) > buprenorphine ($14.91 \pm 0.74\%$) > fentanyl ($10.10 \pm 1.73\%$). On the other hand, methadone (10^{-10}–10^{-5} M) did not significantly modify the ouabain-sensitive Na^+,K^+-ATPase activity ($E_{max} = 5.11 \pm 0.92\%$). These results suggest that Na^+,K^+-ATPase activity is involved in the antinociceptive effects of morphine, levorphanol and buprenorphine, but not in that produced by fentanyl and methadone. Thus, we can conclude that at least two subgroups can be distinguished among the μ-opioid receptor agonists taking into consideration the role of Na^+,K^+-ATPase in their antinociceptive effects.

1. Introduction

Na^+,K^+-ATPase (EC 3.6.1.3) comprises the enzymatic machinery involved in many aspects of neural activity such as restoring the ion gradient disturbed during electrical activity, regulating the resting membrane potential and providing cation gradients that drive transmitter and metabolite uptake processes (Aperia, 2007). Therefore, this enzyme contributes, in part, to regulate the neuronal hyperexcitability triggered by nociceptive stimuli.

Morphine has been shown to stimulate Na^+,K^+-ATPase activity when administered both *in vivo* (Wu et al., 2007, Wu et al., 2006, Sharma et al., 1998) and *in vitro* (Wu et al., 2007, Wu et al., 2006; Masocha et al., 2002). As well, we have demonstrated the implication of cerebral Na^+,K^+-ATPase in the antinociceptive effects of morphine (Masocha et al., 2003; González et al., 2012). One suggested molecular model for the control of Na^+,K^+-ATPase by μ-opioid receptors postulates that acute activation of μ-opioid receptor coupled to Gi/o proteins inhibits adenylyl cyclase and decreases cAMP-dependent protein kinase (PKA) activation, leading to a decrease in the phosphorylation of Na^+,K^+-ATPase, which in turn enhances Na^+,K^+-ATPase activity (Masocha et al., 2002; Therien and Blostein, 2000; Wu et al., 2006).

However, it is not known whether μ-opioid receptor agonists, other than morphine and buprenorphine, have any modulatory effect on the activity of neural Na^+,K^+-ATPase or if the enzyme plays a role in the antinociceptive effects induced by these agonists. This information is of interest because several effects of μ-opioid receptor agonists suggest functional heterogeneity within this group of drugs. For example, Zimmerman et al. (1987) and Adams et al. (1990) have demonstrated that agonists of μ-opioid receptors differ in their intrinsic efficacy (i.e. the amount of receptor occupancy to induce a determined level of response). Ocaña et al. (1995) observed that gliquidone, an ATP-sensitive K^+ channel blocker, antagonised and cromakalim, an opener of K^+

* Correspondence to: Department of Pharmacology and Neurosciences Institute, Faculty of Medicine, University of Granada, Avenida del Conocimiento 11, 18016 Granada, Spain. Tel.: +34 958 243538; fax: +34 958 243537.
E-mail address: lggonzal@ugr.es (L.G. González).
[1] Willias Masocha and Luis G. González contributed equally to this work.

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channels, enhanced the antinociception induced by buprenorphine, morphine and methadone but not that induced by fentanyl and levorphanol. Heyman et al. (1989) and Jiang et al. (1990) reported that [D-Pen^{2,5}]enkephalin enhances the antinociception induced by morphine, normorphine and codeine but not that induced by sufentanil, DAMGO and other μ-opioid receptor agonists. Statile et al. (1988) observed that droperidol enhances the antinociception induced by fentanyl and sufentanil but not that induced by morphine. Finally, Sánchez-Blázquez et al. (1999, 2001) demonstrated that morphine, DAMGO, endomorphin-1, endomorphin-2, heroin, methadone and buprenorphine exhibit different patterns of G protein activation in evoking μ-opioid receptor-mediated supraspinal antinociception.

Taken into account the aforementioned antecedents, the purpose of this study was to evaluate the effects of various μ-opioid receptor agonists on synaptosomal Na^+,K^+-ATPase activity *in vitro* and the effects of a Na^+,K^+-ATPase inhibitor (ouabain) on the antinociception induced by these μ-opioid receptor agonists in order to elucidate the neuronal and functional pattern involved in their antinociceptive effects.

2. Materials and methods

2.1. Animals

Female CD-1 mice (Charles River; Barcelona, Spain), weighing 25–30 g were used for all experiments. The animals were housed in a temperature-controlled room at 22 ± 1 °C, with air exchange every 20 min and an automatic 12 light/dark cycle (lights on from 0800 to 2000 h). They were fed a standard laboratory diet and tap water ad libitum until the beginning of the experiments. All experiments were done during the same period of the day (0900–1500 h) to exclude circadian variations in the pharmacological effects.

The mice were handled in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and the updated Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. The experimental protocol was approved by the Research Ethics Committee of the University of Granada, Spain.

2.2. Drugs

The drugs used as μ-opioid receptor agonists and their suppliers were: morphine hydrochloride (Pub Chem CID: 5464110; General Directorate of Pharmacy and Drugs, Spanish Ministry of Health), buprenorphine hydrochloride (Pub Chem CID: 441364; Laboratorios Dr. Esteve S.A., Spain), fentanyl citrate (Pub Chem CID: 13810; Sigma-Aldrich Química SA, Madrid, Spain), methadone hydrochloride (Pub Chem CID: 14184; Sigma-Aldrich Química SA, Madrid, Spain) and levorphanol tartrate (Pub Chem CID: 5464027; Sigma-Aldrich Química SA, Madrid, Spain). The Na^+,K^+-ATPase inhibitor used was ouabain (octahydrate) (Pub Chem CID: 6364534 Sigma-Aldrich Química SA, Madrid, Spain).

2.3. Drug treatments and assessment of antinociception

For the *in vivo* experiments (assessment of antinociception) all the μ-opioid receptor agonists were dissolved in ultrapure water and injected subcutaneously (s.c.) in a volume of 5 ml/kg. The Na^+,K^+-ATPase inhibitor, ouabain, was dissolved in 1% Tween 80 in ultrapure water and injected intracerebroventricularly (i.c.v.) in a volume of 5 μl per mouse. The control animals received the same volume of vehicle. The s.c. injections were done in the interscapular region. The i.c.v. injections were done in the lateral cerebral ventricle of gently restrained nonanaesthesized mice. Mice were grasped firmly by the loose skin behind the head. Considering the bregma coordinate -where the coronal suture intersects the midline- on the skull, the injection site was 1 mm to the right or left on a line drawn through to the anterior base of the ears (coronal suture). Injections were performed into the right or left ventricle randomly. The drug solution was injected perpendicularly through the skull with a 10 μl Hamilton syringe with a sleeve around the needle (0.4 mm external diameter) to prevent the latter from penetrating more than 3 mm into the skull. After the experiments were done the brain was dissected, a section at the level where the needle penetrated through the cortex was cut from fresh tissue and the trajectory of the injection was observed macroscopically. Results from animals in which the tip of the needle did not reach the lateral ventricle were discarded. To ascertain that the drug were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was 99%.

The antinociceptive effect was evaluated using the tail flick test as previously described by González et al. 2012. Briefly, the animals were restrained in a Plexiglas tube and placed on the tail flick apparatus (LI 7100, Letica, S.A). 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.c.v. injection of ouabain or its solvent and immediately thereafter a s.c. injection of drug tested 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 cut-off time was 10 s.

The area under the curve (AUC) of tail-flick latency against time was calculated for each animal with GraphPad Prism, 2007, *v.* 5.0 software (GraphPad Software Inc.; San Diego, CA, USA). The degree of antinociception was determined according to the formula: % antinociception = $[(AUC_d - AUC_v) / (AUC_{max} - AUC_v)] \times 100$, where the AUC_d and AUC_v are the areas under the curve for drug- and vehicle-treated mice, respectively, and AUC_{max} is the area under the curve of maximum possible antinociception (10 s in each determination).

2.4. Preparation of forebrain pure synaptosomes

Mouse forebrain crude synaptosomal pellets were isolated as previously described in detail by González et al. 2012.

Pure intact synaptosomes were obtained by Percoll density gradient separation. Percoll (Amersham Pharmacia Biotech; Madrid, Spain) stock solution was made by adding 0.5 ml of 2.5 M sucrose to 4.5 ml of original Percoll. Solutions of lower Percoll concentration were prepared by appropriate dilution of the stock solution with medium II [250 mM sucrose; 10 mM HEPES; 3 mM EDTA · 4 Na, pH 7.4]. To prepare the Percoll density gradient 3 ml of 16% Percoll solution was pipetted in the bottom of a 14-ml Ultra-Clear centrifuge tube, then 3 ml of 10% Percoll solution was layered over the 16% Percoll solution, and finally, 3.375 ml of a 7.5% Percoll solution (containing 375 μl of the P_2 pellet solution) was layered over the 10% Percoll solution. All steps were carried out at 4 °C. The tubes were centrifuged at 15000 *g* for 20 min at 4 °C. Synaptosomes banded at 10%:16% Percoll interface were collected with a wide-tip Pasteur pipette. To remove the Percoll from the