Antinociceptive property of the nicotinic agonist AG4 in rodents

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Antinociceptive Property of the Nicotinic Agonist AG-4 in Rodents

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ABSTRACT AG-4 has been characterized as a nicotinic agonist by binding (K_i = 26 ± 1.4 µM) and in vitro functional assays. The antinociceptive effect of AG-4 was examined in mice and rats, using the hot plate, abdominal constriction, and paw-pressure tests. In both species, AG-4 (25–150 µg per mouse icv; 100–150 µg per rat icv) produced significant antinociception which was prevented by mecamylamine (2 mg kg–1 ip) and pempidine (3 mg kg–1 ip), but not by atropine (5 mg kg–1 ip), naloxone (1 mg kg–1 ip) and CGP 35348 (100 mg kg–1 ip). In the antinociceptive dose range, AG-4 did not impair mice motor coordination and spontaneous motility as well as inspection activity. The present results have shown that AG-4 is a compound endowed with antinociceptive properties mediated via nicotinic activation and may be a promising beginning for new nicotinic agonists. Drug Dev. Res. 41:1–9, 1997. © 1997 Wiley-Liss, Inc.

Key Words: analgesia; antinociception; nicotinic agonist

INTRODUCTION

Nicotine is a potent modulator of central nervous system (CNS) function because of its ability to modulate ion flux and neurotransmitter release, which leads to a variety of behavioral effects. Nicotine, in fact, has been shown to produce antinociception in cats [Davis et al., 1983], mice [Mattila et al., 1968; Mansner, 1972; Phan et al., 1973; Aceto et al., 1980; Tripathi et al., 1982], rats [Phan et al., 1973; Sahley and Bernston, 1979; Tripathi et al., 1982; Martin et al., 1983], dogs [Kamerling et al., 1982], rabbits, and hamsters [Mattila et al., 1968]. Evidence for an analgesic effect of nicotine in humans has also been reported [Pomerleau et al., 1984].

AG-4 (2-methyl-5-N,N-dimethylaminomethyl-cyclopentan-1-one methyl iodide AG-4) (Fig. 1) was reported by Giannella et al. [1980] to be agonist for the nicotinic receptors (nAChRs). In fact, the equipotent molar ratio of AG-4 compared to acetylcholine (ACh) is equal to 1.4 in the frog rectus abdomin and greater than 1,000 in the guinea pig ileum. Within the framework of research for new nicotinic agonists, it was decided to use AG-4 to lead to new compounds that would penetrate the brain and be more potent and selective for central nAChRs than the parent compounds. The present work was designed to verify the pharmacological profile of AG-4 that has previously been investigated only on peripheral nAChRs. In order to delineate the pharmacological profile of AG-4, its potential analgesic activity was investigated in the mouse hot plate and abdominal constriction tests and in the rat paw pressure test.
MATERIALS AND METHODS

Animals

Male Swiss albino mice (23–30 g) and Wistar rats (200–300 g) from the Morini (San Polo d’Enza, Italy) breeding farm were used. Fifteen mice and four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1°C with a 12-h light/dark cycle, light at 7 a.m. All experiments were carried out according to the guidelines of the European Community Council for experimental animal care.

Hot Plate Test

The method adopted has been described by O’Callaghan and Holtzman [1975]. Mice were placed inside a stainless steel container, thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshop, Siena, Italy. Reaction times (sec), were measured with a stop-watch before and at regular intervals up to a maximum of 60 min after treatment. The endpoint used was the licking of the fore or hind paws. Those mice scoring below 12 and over 18 sec in the pretest were rejected (30%). An arbitrary cutoff time of 45 sec was adopted.

Abdominal constriction test

Mice were injected ip with a 0.6% solution of acetic acid (10 ml kg⁻¹), according to Koster et al. [1959]. The number of stretching movements was counted for 10 min, starting 5 min after injection. The number of movements was counted for 10 min, starting 5 min after injection.

Paw Pressure Test

The antinociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. [1988]. Threshold pressure was measured before treatment and 15, 30, and 45 min after treatment. Rats scoring <30 g or >85 g in the test prior to drug administration were rejected (25%). An arbitrary cutoff value of 250 g was adopted.

Hole-Board Test

The hole board test consists of a 40-cm-square plane with 16 flush mounted cylindrical holes (diameter 3 cm) distributed 4 x 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and left to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from mid-point to mid-point of opposite sides, thus dividing the plane into 4 equal quadrants, automatically signaled the movement of the animals on the surface of the plane. Miniature photoelectric cells, in each of the 16 holes, recorded the exploration of the holes (head plunging activity) by the mice.

Rotarod test

The apparatus consisted of a base platform and a rotating rod of 3-cm diameter with a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 sec according to Vaught et al. [1985]. The performance time was measured before and 15, 30, and 45 min after treatment.

Binding Assay

Cerebral cortices of male Wistar rats (150–200 g) were dissected on ice. The tissue was homogenized in 50 mM Tris-HCl buffer (pH = 7.4 at 2°C) containing 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 2.5 mM CaCl₂. The homogenate was centrifuged at 40,000 g for 10 min; the pellet was resuspended in ice-cold buffer, recentrifuged and resuspended again in buffer. Binding experiments [Pabreza et al., 1990] with [³H]-cytisine (New England Nuclear, Boston, MA, 39.7 Ci/mm) were performed in 250 µl of buffer containing 2 nM [³H]-cytisine, membranes from 15 mg (wet weight) of tissue and the compounds to be tested. After 75 min of incubation at 2°C, separation of bound from free radioligand was performed by rapid filtration through Whatman GF/C glass fiber filters, which were washed three times with ice-cold buffer, dried and counted in 5 ml of Aquassure (Packard, Downers Grove, IL). Nonspecific undefined binding in the presence of 10 µM (−)-nicotine bitartrate and was, routinely, about 10% of the total binding. Ki values were calculated from Cheng-Prusoff [1973] using 1.5 nM as the Kd for [³H]-cytisine, determined by previous saturation experiments.
**Antinociceptive Effect of AG-4**

**Isolated Guinea Pig Ileum**

The action of AG-4 was examined at neuronal nAChRs; 2-cm segments of the terminal portion of the guinea pig ileum were suspended under 1g resting tension in 10-ml organ baths containing Krebs solution (mM): NaCl (137), KCl (2.7), CaCl2 (1.8), MgCl2 (1.05), NaH2PO4 (0.42), NaHCO3 (11.9), and glucose (5.6). The solution was aerated with gas mixture of 95% O2, 5% CO2, and maintained at 37°C. After 1 h of equilibration, the tissues were exposed to DMPP (1,1-dimethyl-4-phenyl-piperazinium jodid) or AG-4. Concentration response curves were constructed non cumulatively. Tissues were exposed to drugs for 30 s with at least 5 min rest between exposures. Effects of the antagonist hexamethonium 30 µM was investigated after a 30 min equilibration.

**Intracerebroventricular Injection Technique**

Intracerebroventricular (icv) administration was performed under ether anaesthesia using isotonic saline as a solvent, according to the method described by Haley and McCormick [1957]. Briefly, during anaesthesia, mice and rats were grasped firmly by the loose skin behind the head. A 0.4-mm external diameter, a hypodermic needle attached to a 10-µl syringe was inserted perpendicularly through the skull at a depth of no more than 2 mm into the brain of the mouse and 4 mm into the brain of the rat, where 5 µl (mice) or 10 µl (rats) was then administered. The injection site was 1.5 mm (mice) or 2.5 mm (rats) from either side of the midline on a line drawn through to the anterior base of the ears. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice and rats were icv injected with 5–10 µl of diluted 1:10 Indian ink and their brains examined macroscopically after sectioning. Intraplantar injections of carrageenan were performed by injecting 100 µl of a suspension in sterile saline solution of 0.5% carrageenan in the rat hind paw.

**Reagents and Compounds**

The following drugs were used: AG-4 was a kind gift of Professor Giannella, University of Camerino; atropine sulfate, pempidine hydrochloride, naloxone hydrochloride (Sigma, St. Louis, MO); morphine hydrochloride (U.S.L. 10/D, Florence); CGP 35348 and (±)-baclofen (Novartis, Basle, CH); (+)-amphetamine sulfate (Recordati); mecamylamine hydrochloride, McN-A-343 (RB1); DMPP (1,1-dimethyl-4-phenyl-piperazinium jodide), nicotine hydrogentartrate (Fluka); [3H]-cytisine (New England Nuclear): 39.7 Ci/mmol). Other chemicals were of the highest quality commercially available. All drugs were dissolved in isotonic (NaCl 0.9%) saline solution. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg⁻¹ by subcutaneous (sc), intraperitoneal (ip) or 5 µl per mouse by icv injection.

**Statistical Analysis**

All experimental results are given as the mean ± s.e.m. An analysis of variance (ANOVA), followed by Fisher’s PLSD procedure for post-hoc comparison, was used to verify significance between two means. Data were analyzed with the StatView software for the Macintosh (1992). P-values of <0.05 were considered significant.

**RESULTS**

**Binding assay**

The Ki value of AG-4 was 26 ± 1.4 µM (mean ± s.e.m., n = 4) in comparison with nicotine, which had a Ki value of 8.2 ± 0.5 nM at displacing the binding of [3H]-cytisine sites. (Data not shown.)

**Isolated Guinea Pig Ileum**

AG-4 was able to constrict guinea pig isolated ileum fragments with an EC₅₀ of 3.05 µM. This effect was blocked by the nicotinic antagonist hexamethonium 30 µM. The concentration-response curves for AG-4 and DMPP, used as reference drug, are illustrated in Figure 2.

**Antinociceptive Activity of AG-4**

AG-4, as shown in Figure 3, produced a dose-dependent increase in the pain threshold in the mouse hot-plate test after icv (25–150 µg per mouse) administration. The direct injection of AG-4 into the cerebral ventricles...
was needed since this compound is cationic and does not cross the blood brain barrier. The antinociceptive effect of AG-4 peaked 15–30 min after injection and then diminished. The areas under the curve of the antinociception induced by AG-4 (150 µg per mouse icv), nicotine (1.5 µg per mouse icv), morphine (2.0 µg per mouse icv), and McN-A-343 (30 µg per mouse icv) are reported in Figure 4.

Figure 5 illustrates the analgesic effect of AG-4 in the mouse acetic acid abdominal constriction test. In this test AG-4 induced an increase in the pain threshold in a dose-dependent manner starting from the dose of 50 µg per mouse icv.

AG-4 was able to produce an increase in the pain threshold not only in mice but also in rats. In the paw pressure test AG-4 administered icv at the dose of 100 and 150 µg per rat, induced antinociception starting 15 min after injection and persisting up to 45 min (Table 1).

**Antagonism of the AG-4-Induced Antinociception**

In the mouse hot-plate test, the antinociceptive effect of AG-4 (100 µg per mouse icv) was not antagonized by atropine (5 mg kg⁻¹ ip), naloxone (1 mg kg⁻¹ ip) and CGP-35348 (100 mg kg⁻¹ ip) in the mouse hot plate test as reported in Table 2. Conversely, mecamylamine (2 mg kg⁻¹ ip) and pempidine (3 mg kg⁻¹ ip) were able to completely prevent AG-4 antinociception in both hot plate (Table 3) and abdominal constriction tests (Fig. 5). All antagonists were injected 15 min before AG-4, with the exception of CGP 35348, injected 5 min before AG-4.

Figure 6 shows that the doses of 2 mg kg⁻¹ ip and 3 mg kg⁻¹ ip of mecamylamine and pempidine, respectively, were needed to completely antagonize the antinociception induced by nicotine (1.5 µg per mouse icv) and AG-4 (100 µg per mouse icv) without interfering in any way with morphine (2 µg per mouse icv) and baclofen (4 mg kg⁻¹ sc) evoked analgesia.

**Evaluation of the AG-4 Effect on Spontaneous Activity and Motor Coordination**

The motor coordination of mice treated with AG-4 was evaluated by using the rota-rod test (Table 3), while their spontaneous activity and inspection activity were investigated by using the hole-board test (Fig. 7). The rotarod performance of mice treated with 100–150 µg per mouse icv of AG-4, was not impaired in comparison with controls (Table 4). On the contrary, AG-4 administered at higher doses (200 µg per mouse icv) produced a significant impairment of the rota-rod performance (Table 3). The number of falls by control animals progressively decreased at every measurement since the mice learned how to balance on the rotating rod. The spontaneous motil-

ity and exploratory behavior of mice was not modified by treatment with AG-4 (100–150 µg per mouse icv) as revealed by the hole-board test (Fig. 7). In the same experimental conditions, amphetamine (2 mg kg⁻¹ sc) produced an increase of both parameters evaluated (Fig. 7).
Early studies of AG-4 characterized the compound as a nicotinic agonist [Giannella et al., 1980]. Our research confirms and extends this finding. AG-4 showed a moderate affinity for nAChR, labelled with \[^{[3]H}\)-cytisine and, furthermore, was able to constrict the isolated guinea pig ileum.

AG-4 was also able to induce antinociception in laboratory animals. AG-4-induced antinociception was elicited regardless of which noxious stimulus was used: thermal (hot-plate), chemical (abdominal constriction test) and mechanical (paw pressure test). The pharmacological actions of AG-4 were obtained without producing any visible modification of the animals’ gross behavior. Moreover, AG-4 treated mice retained their motor coordination on the rota-rod test, normal spontaneous motility, and exploratory behavior as revealed by the hole-board test. However, at doses three times higher than those effective as analgesic, AG-4 showed marked side effects such as convulsions (data not shown).

AG-4 antinociception was found to be dependent on central nicotinic system activation, since it was prevented by the nicotinic antagonists mecamylamine and pempidine, administered at doses able to antagonize the antinociception induced by nicotine without interfering in any way with morphine and baclofen evoked analgesia.

The involvement of other neurotransmitter systems in AG-4 antinociception can be ruled out since the opioid antagonist naloxone, the muscarinic antagonist atropine and the \(\gamma\)-aminobutyric acid-B (GABA\(_B\) antagonist, CGP-35348, were all unable to prevent the effect of AG-4. The doses and administration schedules of the above-mentioned drugs were ideal for preventing antinociceptions induced, respectively, by morphine [Ghelardini et al., 1992], oxotremorine [Bartolini et al., 1987], and the GABA\(_B\) agonist, baclofen [Malecangio et al., 1991].

AG-4, like nicotine, demonstrated antinociception properties, but with greater efficacy than that exerted by nicotine. Furthermore, AG-4 is endowed with a longer lasting analgesic effect than nicotine even if it starts 10 min later. However, the potency of AG-4 is 100 times lower than that of nicotine and this reflects the lower binding affinity of AG-4 for nicotinic receptors compared to nicotine. The analgesic effect of AG-4 was also compared with that induced by well-known analgesic drugs such as morphine, nicotine, and the selective M\(_1\) antagonist McN-A-343 at the highest doses that did not impair rota-rod performance. By comparing the areas under the curve, the antinociceptive efficacy of AG-4 (150 \(\mu\)g per mouse icv) was almost equal to those exerted by morphine (2.0 \(\mu\)g per mouse icv) and McN-A-343 (30 \(\mu\)g per mouse icv), but greater than that induced by nicotine (1.5 \(\mu\)g per mouse icv).

In summary, our results have shown that the new nicotinic agonist AG-4 is able to produce antinociception...
### TABLE 1. Antinociception Exerted by AG-4 in the Paw-Pressure Test in the Rat and Antagonism by Mecamylamine†

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment (icv)</th>
<th>Dose (µg/mouse)</th>
<th>Paw pressure (g)</th>
<th>Before pretreatment</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>After treatment</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>58.9 ± 4.2</td>
<td>13.6 ± 4.2</td>
<td>60.2 ± 3.1</td>
<td>57.8 ± 3.2</td>
<td>60.3 ± 3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ml kg⁻¹ ip</td>
<td>AG-4</td>
<td>60.6 ± 3.0</td>
<td>55.6 ± 3.8</td>
<td>68.7 ± 4.4</td>
<td>56.7 ± 4.2</td>
<td>55.6 ± 3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>AG-4</td>
<td>100</td>
<td>112.6 ± 5.1*</td>
<td>107.8 ± 4.2*</td>
<td>73.5 ± 4.5**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>Saline</td>
<td>58.4 ± 3.5</td>
<td>60.3 ± 4.1</td>
<td>55.8 ± 3.8</td>
<td>64.1 ± 3.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg kg⁻¹ ip</td>
<td>AG-4</td>
<td>100</td>
<td>67.2 ± 5.4†</td>
<td>66.2 ± 4.3†</td>
<td>62.1 ± 4.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†The number of rats is shown in parentheses.
*P < 0.01.
**P < 0.05 in comparison with saline–saline.
‡P < 0.01 versus saline–AG-4.

### TABLE 2. Effects of Atropine, Naloxone, CGP-35348, Mecamylamine, and Pempidine on Antinociception Caused by AG-4 in the Mouse Hot Plate Test†

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment (icv)</th>
<th>Dose (µl)</th>
<th>Licking latency (s)</th>
<th>Before pretreatment</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>After treatment</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>5.6 ± 0.7</td>
<td>13.6 ± 0.9</td>
<td>14.4 ± 1.0</td>
<td>13.9 ± 0.9</td>
<td>15.1 ± 0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ml kg⁻¹ ip</td>
<td>AG-4</td>
<td>100</td>
<td>14.1 ± 0.8</td>
<td>27.5 ± 1.4*</td>
<td>26.7 ± 1.7*</td>
<td>22.4 ± 1.8*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>AG-4</td>
<td>100</td>
<td>13.8 ± 0.9</td>
<td>26.7 ± 1.4*</td>
<td>21.9 ± 2.1</td>
<td>17.8 ± 2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naloxone</td>
<td>AG-4</td>
<td>100</td>
<td>13.9 ± 0.8</td>
<td>13.5 ± 1.2</td>
<td>14.6 ± 1.7</td>
<td>14.3 ± 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mg kg⁻¹ ip</td>
<td>CGP-35348</td>
<td>100</td>
<td>14.2 ± 0.9</td>
<td>27.3 ± 2.1*</td>
<td>25.8 ± 2.2*</td>
<td>19.4 ± 1.9**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg kg⁻¹ ip</td>
<td>AG-4</td>
<td>100</td>
<td>14.5 ± 0.8</td>
<td>25.1 ± 1.8*</td>
<td>27.8 ± 2.0*</td>
<td>21.5 ± 1.4*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>Saline</td>
<td>100</td>
<td>14.4 ± 1.0</td>
<td>14.9 ± 1.7</td>
<td>14.1 ± 1.6</td>
<td>14.7 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mg kg⁻¹ ip</td>
<td>CGP-35348</td>
<td>100</td>
<td>13.5 ± 0.9</td>
<td>15.6 ± 2.0†</td>
<td>15.8 ± 1.4†</td>
<td>16.3 ± 1.3†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pempidine</td>
<td>AG-4</td>
<td>100</td>
<td>15.0 ± 0.6</td>
<td>14.7 ± 1.6</td>
<td>14.9 ± 1.7</td>
<td>15.3 ± 1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 mg kg⁻¹ ip</td>
<td>AG-4</td>
<td>100</td>
<td>14.3 ± 0.8</td>
<td>16.8 ± 1.4†</td>
<td>17.3 ± 2.0†</td>
<td>15.9 ± 1.5†</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†The number of mice is shown in parentheses. The number of mice ranged from 8 to 18, with the exception of saline–saline, which numbered 24.
*P < 0.01.
**P < 0.05 in comparison with saline–saline.
†P < 0.01 versus saline–AG-4.

### TABLE 3. Effect of AG-4 in the Mouse Rota-rod Test†

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>No. of falls (30s)</th>
<th>Before treatment</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5 µl</td>
<td>3.6 ± 0.3</td>
<td>2.4 ± 0.3</td>
<td>1.8 ± 0.2**</td>
<td>1.3 ± 0.1***</td>
<td>1.3 ± 0.1**</td>
<td></td>
</tr>
<tr>
<td>AG-4</td>
<td>100 µg</td>
<td>3.2 ± 0.5</td>
<td>2.6 ± 0.3</td>
<td>2.0 ± 0.3**</td>
<td>1.5 ± 0.3***</td>
<td>1.6 ± 0.2**</td>
<td></td>
</tr>
<tr>
<td>AG-4</td>
<td>150 µg</td>
<td>3.4 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>2.2 ± 0.3**</td>
<td>1.8 ± 0.4**</td>
<td>1.2 ± 0.1**</td>
<td></td>
</tr>
<tr>
<td>AG-4</td>
<td>200 µg</td>
<td>3.5 ± 0.4</td>
<td>3.9 ± 0.5*</td>
<td>4.0 ± 0.5*</td>
<td>3.4 ± 0.4*</td>
<td>2.3 ± 0.2**</td>
<td></td>
</tr>
</tbody>
</table>

†Each value represents the mean of at least 8 mice.
*P < 0.01 in comparison with saline controls.
**P < 0.01 in comparison with the respective pre-test value.
Fig. 6. Effect of mecamylamine (A) and pempidine (B) administered ip on antinociception induced by nicotine (1.5 µg per mouse icv), AG-4 (100 µg per mouse icv), morphine (2 µg per mouse icv), and baclofen (4 mg kg⁻¹ sc) in the mouse hot-plate test. Mecamylamine and pempidine were injected ip 10 min before nicotine and AG-4 and 5 min before morphine and baclofen. Nociceptive responses were recorded 10 min after nicotine injection, 15 min after morphine and AG-4 administration and 30 min after baclofen injection. Numbers inside the columns indicate the number of mice. Vertical lines show s.e.m. *P < 0.01 in comparison with saline-treated controls. oP < 0.01 versus nicotine or AG-4 treated mice.
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