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Blockade of clomipramine and amitriptyline analgesia by an antisense oligonucleotide to mKv1.1, a mouse *Shaker*-like K^+ channel

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Abstract

The effect of an antisense oligonucleotide to the K^+ channel coding mKv1.1 mRNA on antinociception induced by the tricyclic antidepressants, clomipramine (20–35 mg kg^{-1} s.c.) and amitriptyline (10–25 mg kg^{-1} s.c.), was investigated in the mouse hot-plate test. Antisense oligonucleotide (0.5-1.0-2.0-3.0 nmol per i.c.v. injection) produced a dose-dependent inhibition of clomipramine and amitriptyline antinociception 72 h after the last i.c.v. injection. The sensitivity to both analgesic drugs returned 7 days after antisense oligonucleotide injection, indicating the absence of irreversible damage or toxicity. Treatment with a degenerated oligonucleotide did not modify the clomipramine- and amitriptyline-induced antinociception in comparison with that in naive (unpretreated controls), vector and saline i.c.v.-injected mice. A quantitative reverse transcription-polymerase chain reaction (RT-PCR) study demonstrated a reduction in mRNA levels only in the antisense oligonucleotide treated group. Antisense oligonucleotide, degenerated oligonucleotide or vector pretreatment, in the range of doses used, did not produce any behavioural impairment as revealed by the mouse rotarod and hole-board tests. The present results indicate that modulation of the mKv1.1 K^+ channel plays an important role in the central analgesia induced by the tricyclic antidepressants, clomipramine and amitriptyline. © 1997 Elsevier Science B.V.

Keywords: Antisense oligonucleotide; K^+ channel; mKv1.1; Analgesia; Antinociception; Clomipramine; Amitriptyline; Tricyclic antidepressant; (Mouse)

1. Introduction

The tricyclic antidepressants have been used for many years to suppress certain types of pain in humans. These types include diabetic neuropathy, postherpetic neuralgia, headaches, arthritis, chronic back pain, cancer pain, phantom limb pain. Tricyclic antidepressants are also analgesics in laboratory animals (Murua and Molina, 1991; Goldstein et al., 1990; Acton et al., 1992). The analgesic effect of tricyclic antidepressant drugs seems to be independent of their antidepressant activity since the doses used for analgesia are lower than those considered effective in the treatment of depression (Acton et al., 1992).

The serotoninergic neurones of the dorsal raphe nucleus innervate numerous areas of the brain and spinal cord such as the hippocampus and the periaqueductal gray neurones (Pazos and Palacios, 1985), regions involved in a variety of diverse physiological functions including pain modulation (Besson and Chaouch, 1987). Clomipramine and amitriptyline, as well as other tricyclic antidepressants, are inhibitors of serotonin reuptake. The subsequent increase of endogenous serotonin in the synaptic cleft is responsible for the increase in the pain threshold in mice directly (Galeotti et al., 1995), or through activation of the opioid system (Sacerdote et al., 1987). Furthermore, clomipramine analgesia has been reported to underlie the activation of a signal transduction mechanism operated by pertussis toxin-sensitive G-proteins ($G_{i/o}$ proteins) (Galeotti et al., 1996).

The stimulation of 5-HT receptors can affect several K^+ channels in central neurones as documented in biochemical and electrophysiological studies (Bobker and Williams, 1990). Neurones possess a wide variety of kinetically distinct K^+ channels. Many of these are voltage-dependent (Catterall, 1988; Jan and Jan, 1989), others can be modulated by neurotransmitters interacting with G-

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protein-coupled receptors (Brown and Birnbaumer, 1990; Brown, 1990). G proteins can modulate different types of K^+ channels through a direct effect on the ion channel or through an enzymatic step leading to the generation of cytoplasmic second messengers (Brown, 1990; Hille, 1994).

Since little is known about the intracellular effectors involved in the mechanism of action of tricyclic antidepressants, we decided to investigate the role of K^+ channels in the clomipramine and amitriptyline enhancement of the pain threshold. The first mammalian K^+ channel genes were isolated from mouse brain (Tempel et al., 1988) and rat brain (Baumann et al., 1988), based on the similarity of these genes to the *Drosophila Shaker* K^+ channel genes (Jan and Jan, 1989; Salkoff et al., 1992). These genes were designated Kv1.1 to indicate their ionic selectivity, voltage sensitivity, subfamily membership and order of isolation (Chandy, 1991). Functional expression of the mouse Kv1.1 gene in *Xenopus* oocytes (Hopkins and Tempel, 1992) and in Chinese hamster ovary cells (Robertson and Owen, 1993) demonstrated that mKv1.1 (previously MBK1) generates, in contrast to the fast transient currents (I_A) elicited by the *Shaker* transcript injection, rapidly activating outward currents (I_K), characteristic of delayed rectifier K^+ channels. The mKv1.1 K^+ channel proteins are widely distributed in the central nervous system (CNS), including areas involved in the regulation of pain perception, and are able to modulate neuronal function (Wang et al., 1994). A specific antisense oligodeoxyribonucleotide targeting the translation start region of the mKv1.1 mRNA was designed. The effect of intracerebroventricular injection of the antisense oligodeoxyribonucleotide, in comparison with that of a degenerated oligodeoxyribonucleotide, on the antinociception induced by the antidepressant drugs, clomipramine and amitriptyline, was evaluated in mice.

We also investigated whether amitriptyline analgesia, like clomipramine analgesia, was prevented by intracerebroventricular administration of pertussis toxin.

2. Materials and methods

2.1. Animals

Male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) were used. The mice were housed 15 per cage. The cages were placed in the experimental room 24 h before the test for adaptation. 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 on at 7 a.m.

2.2. Antisense oligonucleotides

24mer phosphodiester oligonucleotides (ODNs) were capped by a terminal phosphorothioate double substitution and purified by high-performance liquid chromatography (HPLC; Genosys, The Woodlands, TX, USA). The antisense oligonucleotide (5′-CGA CAT CAC CGT CAT GAT GAA AGC-3′) was designed by targeting the 5′ portion of the murine Kv1.1 mRNA, residues 575–598 of the published cDNA sequence (Chandy et al., 1990). A fully degenerated 24mer oligonucleotide was used as control.

2.3. Administration of antisense oligonucleotide

Mice were randomly assigned to an antisense oligonucleotide, degenerated oligonucleotide, vector (DOTAP; *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate), saline or naive group. 100, 200, 400 and 600 μM oligonucleotides were preincubated at 37°C for 30 min with 13 μM DOTAP. Each group received a single intracerebroventricular (i.c.v.) injection on days 1, 4 and 7.

2.4. Administration of pertussis toxin

Mice were randomly assigned to a vehicle (water solution containing 0.01 M sodium phosphate buffer, pH 7.0, with 0.05 sodium chloride) or a pertussis toxin group (0.25 μg per mouse). Naive animals did not receive any pretreatment whereas vehicle and pertussis toxin groups received a single intracerebroventricular (i.c.v.) injection on day 1.

2.5. Intracerebroventricular injection technique

Intracerebroventricular (i.c.v.) 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 were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl was then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle randomly. To ascertain that the oligonucleotides were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of 1:10 diluted India ink and their brains were examined macroscopically after sectioning.

2.6. Behavioural tests

2.6.1. Hot plate test

The method adopted was 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 (s), were measured with a stopwatch before and 15, 30 and 45 min after treatment. The endpoint used was licking of the forepaws or hind paws. Mice

scoring below 12 and over 18 s in the pretest were not used (30%). An arbitrary cut-off time of 45 s was adopted.

The analgesic effect of amitriptyline on the pertussis toxin-treated group was evaluated 11 days after pertussis toxin injection.

Following the above-mentioned pretreatment schedule with the antisense oligonucleotide, the antinociceptive effect of clomipramine and amitriptyline was evaluated 72 h and 7 days after the last i.c.v. injection. The percentage of the maximum analgesic effect was evaluated as follows:

% of maximum analgesic effect

$$= \frac{(\text{antisense ODN} - \text{pretest}) \times 100}{\text{degenerated ODN} - \text{pretest}}$$

2.6.2. *Hole-board test*

The hole board test setup utilizes a 40 cm square plane with 16 flush-mounted cylindrical holes (diameter 3 cm) distributed 4 by 4 in an equidistant, grid-like manner. The mice were placed in the center of the board one by one and left to move about freely for a period of 5 min each. Two photoelectric beams, 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. The test was performed 72 h after the last i.c.v. injection of antisense oligonucleotide, degenerated oligonucleotide or vector.

2.6.3. *Rotarod test*

The apparatus consists of a base platform and a rotating rod of 3 cm diameter with a non-skid 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-rotation speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s, according to Vaught et al. (1985). Performance time was measured before and 15, 30 and 45 min after subcutaneous administration of saline. The test was performed 72 h after the last i.c.v. injection of antisense oligonucleotide, degenerated oligonucleotide or vector.

2.7. *In vitro studies*

2.7.1. *RT-PCR-based analysis of mKv1.1 mRNA*

Mouse brain levels of Kv1.1 mRNA were determined by a quantitative reverse transcription-polymerase chain reaction (RT-PCR) method (Quattrone et al., 1995a,b). Briefly, 72 h after the last i.c.v. injection of vector, antisense oligonucleotide or degenerated oligonucleotide, between 3 and 5 mice per group were killed and their brains were rapidly removed and stored (−80°C). Frozen brain samples (0.2–0.3 g w.w.) were homogenized in 3 volumes of RNAzol in order to extract total RNA according to the manufacturer's instructions. RNA was treated with RQ1 RNase-free DNase, purified by ethanol precipitation, dissolved in water containing an RNase inhibitor (RNasin at 1 u/ml) and reversely transcribed to cDNA using random hexamers. Kv1.1 amplimers were 5′-GCT CTC TCC TGG CCT CCT-3′ and 5′-GTT TCG AAG CGC AGC CCG-3′, residues 544–561 and 715–732 respectively, according to the Kv1.1 cDNA published sequence (Chandy et al., 1990). β-Actin was used as internal standard gene. PCR cycles consisted of 1-min denaturation at 92°C, 1-min annealing at 56°C and 1-min extension at 72°C for 30 cycles. Amplification products were first identified by sequencing then quantified by densitometry.

2.8. *Reagents and drugs*

Oligonucleotides used for the antisense strategy, and specific primers or hexamers used for RT-PCR analysis were from Genosys. DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate) was from Boehringer-Mannheim (Mannheim, Germany). RNAzol was from Cynna Biotecx (Houston, TX, USA); RQ1 RNase-free DNase, RNase ONE, RNasin, Mo-MLV reverse transcriptase and fmol sequencing kit were from Promega (Madison, WI, USA); *Taq* polymerase was from Perkin-Elmer-Cetus (Emeryville, CT, USA). The following drugs were used: clomipramine (Anafranil, Ciba Geigy, Basel, Switzerland), amitriptyline (Sigma, St. Louis, MO, USA), D-amphetamine (De Angeli), pertussis toxin (Research Biochemicals International, Natick, MA, USA).

Analgesic drugs and D-amphetamine were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Antisense and degenerated oligonucleotides were dissolved in the vector (DOTAP) at least 30 min before injection. 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 (s.c.) injection or 5 μl per mouse by i.c.v. injection.

2.9. *Sequence and statistical analysis*

Sequences of Kv genes were from the GenBank database. Sequence comparisons of both antisense oligonucleotide and RT-PCR primers with the database were carried out using the FASTA program. All experimental results are given as the means ± S.E.M. An analysis of variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) procedure for post-hoc comparison, was used to verify the significance of differences between two means of behavioural results. Data were analysed with the StatView software for the Macin-

tosh (1992). The statistical significance of RT-PCR was obtained using Student's *t*-test; P values of less than 0.05 were considered significant.

3. Results

3.1. Design of oligonucleotides

Considering that the translation start sites of mRNAs are particularly sensitive to antisense oligonucleotide action (Goodchild, 1989; Stein and Cheng, 1993), we compared the sequence of this site to the murine Kv1.1 cDNA and other known K^+ channel coding genes, in order to design an effective and specific anti-mouse Kv1.1 antisense oligonucleotide. As summarised in Table 1, we noted that the 24 bp segment 5′-GCT TTC ATC ATG ACG GTG ATG TCG-3′ (residues 575–598 of the published mouse Kv1.1 cDNA sequence; Chandy et al., 1990), has a low sequence homology even with the nearest members of the *Shaker*-like subfamily (54% with Kv1.2, Kv1.3, Kv1.4) and is almost totally unrelated to members of other Kv gene subfamilies. We therefore designed an antisense oligonucleotide which is complementary to this Kv1.1 mRNA segment and is most probably unable to target other mouse Kv mRNAs. Moreover, this antisense oligonucleotide has terminal GCs at both 5′- and 3′-end, known for enhancing the binding affinity of antisense oligonucleotide/mRNA heteroduplexes. Considering the described sequence-independent, non-antisense effects of oligonucleotides (Storey et al., 1991; Gao et al., 1992; Blagosklonny and Neckers, 1994; Schick et al., 1995), we designed a fully degenerated phosphodiester-phosphorotioate capped oligonucleotide as the most suitable control for these potentially confusing effects. The fully degenerated 24mer is a collection of about 3×10^{14} different molecular species, so that for the nanomolar-micromolar range concentrations achieved in in vitro antisense experiments for this degenerated control, every species, i.e. every oligonucleotide of defined sequence, is present at the site of action at a sub-attomolar concentration which is totally insufficient to achieve any antisense, or generally sequence-dependent, cellular effect. Therefore, if oligonucleotide i.c.v. administration per se had yielded any biological response, this would have been present in degenerated oligonucleotide-treated controls.

3.2. Effect of pertussis toxin on amitriptyline antinociception

Pertussis toxin, administered at the dose of 0.25 μg per mouse i.c.v. 11 days prior to the test, led to a loss of the antinociceptive effect of amitriptyline (20 mg kg^{-1} s.c.). This antagonism was revealed by using the hot-plate test as shown in Table 2.

3.3. Effect of antisense oligonucleotide on clomipramine and amitriptyline antinociception

The effects produced by the antisense oligonucleotide to the mKv1.1 gene on clomipramine (20–35 mg kg^{-1} s.c.) and amitriptyline (10–25 mg kg^{-1} s.c.) analgesia were evaluated by using the mouse hot-plate test.

72 h after pretreatment with antisense oligonucleotide (0.5 nmol per i.c.v. injection) the mice had no statistically significant reduction in clomipramine (25 mg kg^{-1} s.c.; Fig. 1A) and amitriptyline (20 mg kg^{-1} s.c.; Fig. 2A) analgesia compared with vector and degenerated oligonucleotide treated groups. On the other hand, at the dose of 1.0 and 2.0 nmol per i.c.v. injection, the antisense oligonucleotide dose dependently prevented the analgesic effect of clomipramine (Fig. 1B and C) and, at the dose of 1.0 and 3.0 nmol per i.c.v. injection, dose dependently reduced amitriptyline antinociception (Fig. 2B and C). These ant-

Table 1
Homology of aODN-targeted sequence in mouse Kv1.1 mRNA both with other genes of the *Shaker*-like subfamily and with Kv genes of other subfamilies (translation start codons are underlined; base identity are bold)

Gene target	Sequence	% homology	Acc. number
Kv1.1	**GCUUUCAUCAUGACGGUGAUGUCG**		M30439
Shaker-related			
Kv1.2	**GC**CCCA**AU**U**AUGAC**A**GUG**GCUA**C**C	54	M30440
Kv1.3	C**C**GCCAGA**CAUGAC**C**GUG**G**UG**C**C**C	54	M30441
Kv1.4	A**C**CAC**CA**C**CAUG**GA**GGUG**GC**G**AU**G**	54	U03723
Kv1.5	C**C**CCGG**A**C**CAUG**GAGA**U**CUCCCU**G**	33	L22218
Kv1.7	CGAGGUGA**CAUGA**GAUC**G**GA**G**AAA	29	M96688
Shab-related			
Kv2.1β	**G**GCGC**C**GCG**AUG**GGCCAAGG**G**GAC	25	L48983
Shaw-related			
Kv3.1	**G**GCG**C**CGCG**AUG**GGCCAAGG**G**GAC	25	Y07521
Kv3.3	**GC**GG**U**GGC**CAUG**GG**G**CG**G**CACGGC	37	X60796

Table 2
Effect of pertussis toxin (PTX) pretreatment on amitriptyline-induced antinociception in the mouse hot-plate test

Pretreatment	Treatment	Licking latency (s)			
		Before treatment	After treatment		
			15 min	30 min	45 min
Vehicle	Saline	15.9 ± 0.8	15.2 ± 0.7	15.0 ± 0.9	14.4 ± 0.8
Vehicle	Amitriptyline	14.2 ± 0.4	25.2 ± 1.2	29.2 ± 2.5	20.7 ± 2.0
PTX 0.25 μg	Saline	14.5 ± 1.2	13.4 ± 1.1	13.3 ± 1.3	13.7 ± 1.3
PTX 0.25 μg	Amitriptyline	14.2 ± 0.7	15.7 ± 1.2 [b]	15.0 ± 1.3 [b]	14.4 ± 1.2 [a]

The test was performed 11 days after a single i.c.v. injection of vehicle or pertussis toxin (0.25 μg per mouse). Saline and amitriptyline (20 mg kg^{-1}) were administered s.c.; values are means ± S.E.M.; there were 8–22 mice per group. [a] $P < 0.05$, [b] $P < 0.01$ in comparison with vehicle-amitriptyline-treated mice.

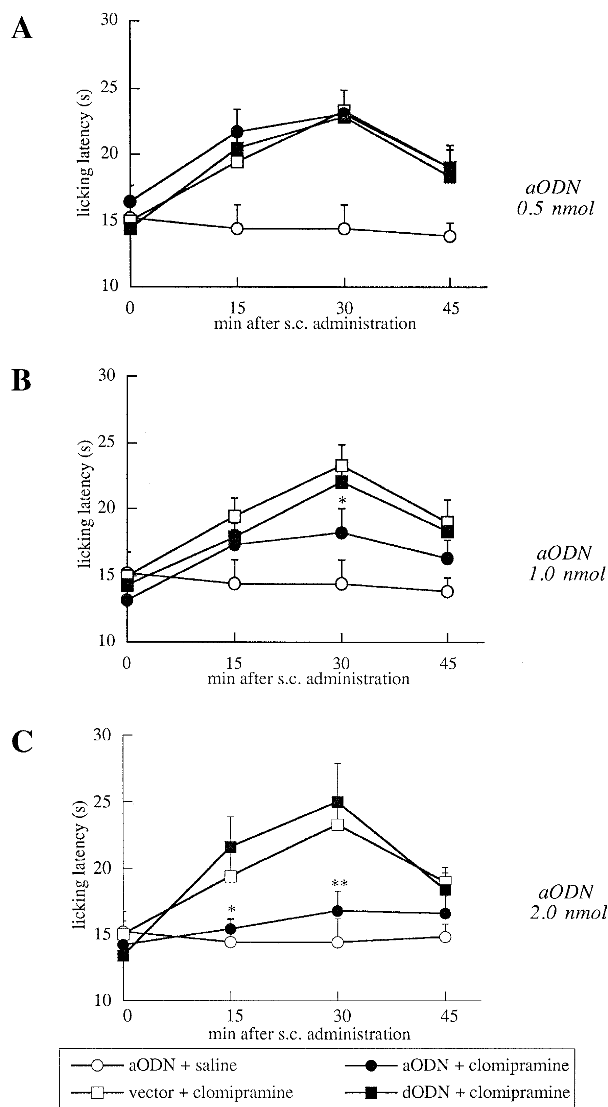


Fig. 1. Prevention of clomipramine (25 mg kg^{-1} s.c.)-induced antinociception by pretreatment with an antisense ODN (aODN) to mKv1.1 gene in the mouse hot-plate test. Mice were i.c.v. injected with vector, antisense ODN or degenerated ODN (dODN) at the dose of 0.5 (A), 1.0 (B) and 2.0 nmol per injection (C) on days 1,4 and 7. The hot-plate test was performed 72 h after the last i.c.v. injection. Vertical lines give S.E.M.; there were 10–17 mice per group. * $P < 0.05$, ** $P < 0.01$ in comparison with degenerated ODN + clomipramine-treated mice.

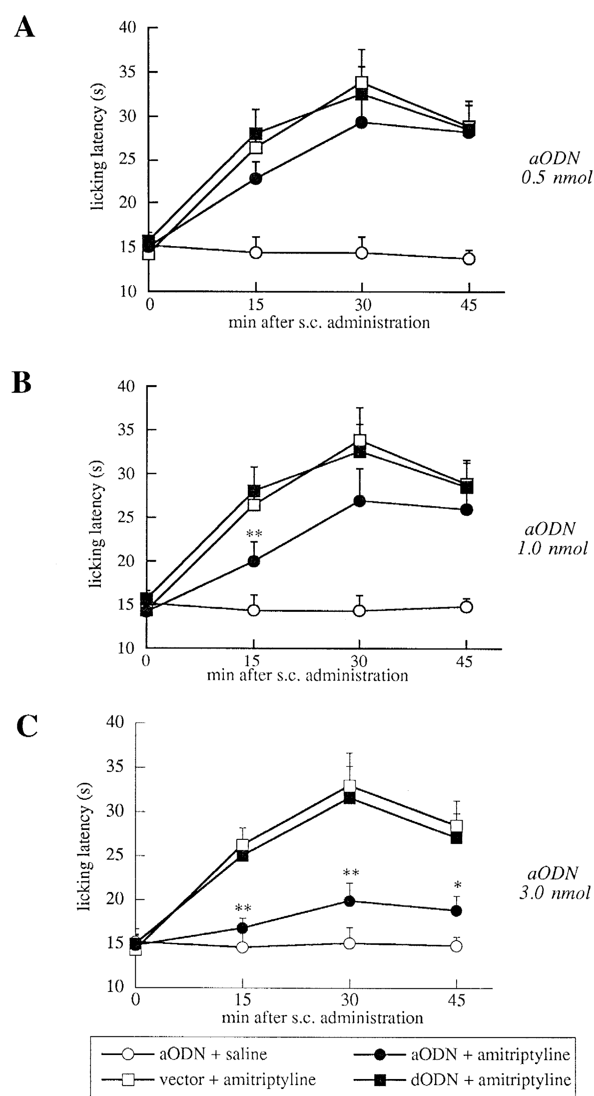
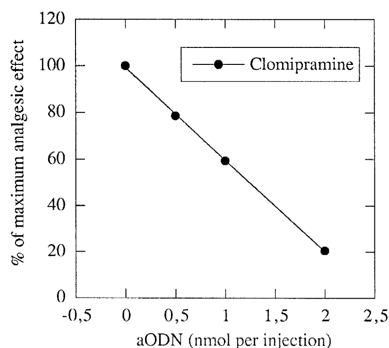


Fig. 2. Prevention of amitriptyline (20 mg kg^{-1} s.c.)-induced antinociception by pretreatment with an antisense ODN (aODN) to mKv1.1 gene in the mouse hot-plate test. Mice were i.c.v. injected with vector, antisense ODN or degenerated ODN (dODN) at the dose of 0.5 (A), 1.0 (B) and 3.0 nmol per injection (C) on days 1, 4 and 7. The hot-plate test was performed 72 h after the last i.c.v. injection. Vertical lines give S.E.M.; there were 9–17 mice per group. * $P < 0.05$ in comparison with degenerated ODN + amitriptyline treated mice.

panel A



panel B

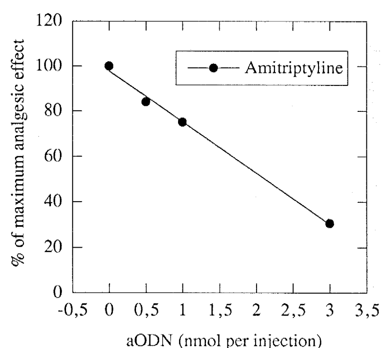


Fig. 3. Effect of increasing concentrations of an antisense ODN (aODN) to the mKv1.1 gene on clomipramine- (25 mg kg^{-1} s.c.) (A) and amitriptyline- (20 mg kg^{-1} s.c.) (B) induced antinociception in the mouse hot-plate test. Mice received a single i.c.v. injection of antisense ODN (0.5-1.0-2.0-3.0 nmol per injection) on days 1, 4 and 7. The hot-plate test was performed 72 h after the last i.c.v. injection. The analgesic effect was detected 30 min after clomipramine and amitriptyline administration. Each point represents the mean for at least 10 mice.

agonistic effects were detected 72 h after the end of the antisense oligonucleotide pretreatment. The pretreatment with the degenerated oligonucleotide never modified clomipramine and amitriptyline induced antinociception in comparison with vector i.c.v.-injected mice as shown in Figs. 1 and 2, respectively.

The dose-dependent inhibition of clomipramine and amitriptyline antinociception produced by increasing concentrations of antisense oligonucleotide is shown in Fig. 3A and B, respectively. The percentage of the maximum analgesic effect corresponded with the maximum effect of clomipramine and amitriptyline occurring 30 min after administration.

Both clomipramine (20–35 mg kg^{-1} s.c.) and amitriptyline (10–25 mg kg^{-1} s.c.) produced dose-dependent antinociception (Fig. 4). In order to restrict the observation to the range of doses of clomipramine and amitriptyline with analgesic activity and devoid of other behavioral effects, we tested clomipramine and amitriptyline at doses that did not impair motor coordination (data not shown). Pretreatment with antisense oligonucleotide (1.0–3.0 nmol per i.c.v. injection) prevented the antinociception induced by increasing concentrations of clomipramine (Fig. 4, panel A) and amitriptyline (Fig. 4, panel B) to different degrees depending on the dose of analgesic drug that displaced to the right the clomipramine (panel A) and amitriptyline (panel B) dose-response line. The licking latency values reported in Fig. 4 were evaluated in relation to the maximum analgesic effect of clomipramine and amitriptyline (30 min after administration).

The prevention of clomipramine and amitriptyline analgesia produced by antisense oligonucleotide at, respectively, 2.0 nmol and 3.0 nmol per i.c.v. injection, disappeared 7 days after the end of the antisense oligonucleotide pretreatment (Fig. 5).

The antisense oligonucleotide pretreatment did not reduce the pain threshold in mice, having no hyperalgesic effect at both 72 h (Figs. 1 and 2) and 7 days (Fig. 5) after the last i.c.v. injection of antisense oligonucleotide.

Repeated i.c.v. injections of vector or saline did not modify the sensitivity of animals to the analgesic treat-

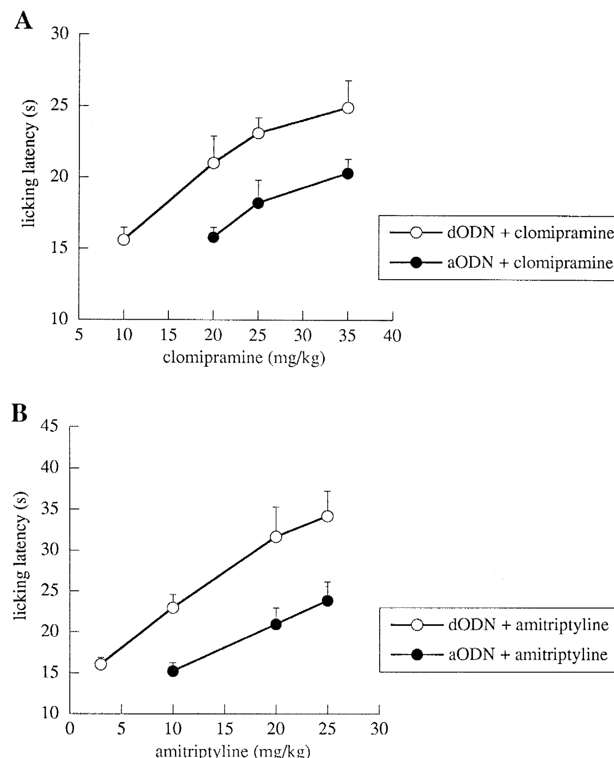


Fig. 4. Effect of i.c.v. pretreatment with an antisense ODN (aODN) to mKv1.1 gene or degenerated ODN (dODN) on the antinociceptive effect produced by increasing concentrations of clomipramine (20–35 mg kg^{-1} s.c., panel A) and amitriptyline (10–25 mg kg^{-1} s.c., panel B) in the mouse hot-plate test. Mice received a single i.c.v. injection of aODN or dODN at the dose of 1.0 nmol (panel A) or 3.0 nmol (panel B) per injection on days 1, 4 and 7. The hot-plate test was performed 72 h after the last i.c.v. injection. The evaluation of the analgesic effect was carried out 30 min after administration of clomipramine and amitriptyline. Vertical lines give S.E.M.; each point represents the mean for at leas 9 mice.

Table 3
Comparison between vector (DOTAP) and saline pretreatment on clomipramine- and amitriptyline-induced antinociception in the mouse hot-plate test

Pretreatment nmol per mouse i.c.v.	Treatment mg kg^{-1} s.c.	Licking latency in mice (s)			
		Before treatment	After treatment		
			15 min	30 min	45 min
Vector	Saline	16.0 ± 1.8	14.8 ± 1.0	15.0 ± 1.0	14.6 ± 0.7
Saline	Saline	15.9 ± 1.6	14.1 ± 1.0	14.7 ± 1.1	14.3 ± 0.9
–	Saline	14.2 ± 0.9	14.2 ± 1.1	15.6 ± 0.8	15.1 ± 0.9
Vector	Clomipramine 25	15.0 ± 1.8	19.4 ± 1.4 [a]	23.3 ± 1.5 [b]	19.0 ± 1.7 [a]
Saline	Clomipramine 25	15.4 ± 1.4	22.6 ± 2.9 [b]	24.0 ± 3.6 [b]	19.6 ± 2.8 [a]
–	Clomipramine 25	15.0 ± 0.9	19.8 ± 1.4 [b]	24.5 ± 2.3 [c]	20.5 ± 1.3 [b]
Vector	Amitriptyline 20	14.3 ± 0.8	26.4 ± 2.0 [c]	33.9 ± 3.6 [c]	27.9 ± 1.5 [c]
Saline	Amitriptyline 20	12.2 ± 0.4	25.2 ± 1.2 [c]	30.2 ± 2.5 [c]	24.7 ± 2.0 [c]
–	Amitriptyline 20	13.0 ± 0.6	26.7 ± 0.9 [c]	29.6 ± 1.7 [c]	24.6 ± 1.9 [b]

Mice were injected i.c.v. on days 1, 4 and 7 with saline or vector. Non-pretreated (naive) animals did not receive any i.c.v. injection. The experiment was performed 72 h after the last i.c.v. injection. There were 8–21 mice per group. [a] $P < 0.01$, [b] $P < 0.01$, [c] $P < 0.001$ versus corresponding control mice.

ments in comparison with that of unpretreated (naive) mice (Table 3).

3.4. Effect of antisense oligonucleotide on mouse behaviour

The motor coordination of mice pretreated with antisense oligonucleotide (2.0 nmol per injection) or degenerated oligonucleotide (2.0 nmol per injection) was evaluated by using the rotarod test 72 h after the last i.c.v. injection. The motor coordination of both antisense oligonucleotide- and degenerated oligonucleotide-treated groups was not impaired when compared with that of vector-treated mice, since each group progressively reduced its number of falls (Table 4), because mice learned how to balance on the rotating rod.

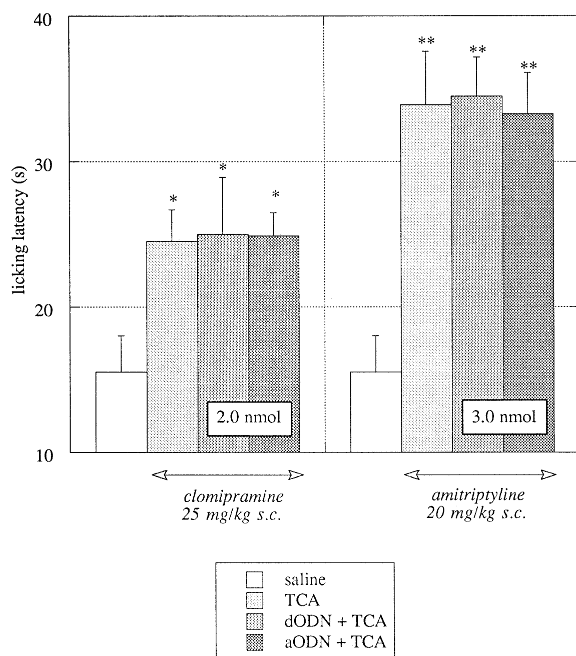


Fig. 5. Lack of effect of an antisense ODN (aODN) to the mKv1.1 gene on the tricyclic antidepressant drugs (TCA) clomipramine (25 mg kg^{-1} s.c.) and amitriptyline (20 mg kg^{-1} s.c.) induced antinociception 7 days after the end of the antisense ODN treatment. Mice were injected with vector, antisense ODN or degenerated ODN (dODN) at the dose of 2.0 or 3.0 nmol per injection on days 1, 4 and 7. Modification of the pain threshold was evaluated by using the mouse hot-plate test. The licking latency was detected 30 min after clomipramine or amitriptyline administration. Vertical lines give S.E.M.; there were 9–12 mice per group. * $P < 0.01$, ** $P < 0.001$ in comparison with saline-treated mice.

The spontaneous motility and inspection activity of mice were not modified by pretreatment with antisense oligonucleotide (2.0 nmol per injection) or degenerated oligonucleotide (2.0 nmol per injection), in comparison with vector-treated mice, as revealed by the hole-board test (Fig. 6). The test was performed 72 h after the end of the antisense oligonucleotide pretreatment. Under the same experimental conditions D-amphetamine (1 mg kg^{-1} s.c.), used as a reference drug, increased both motility and inspection activity (Fig. 6).

3.5. Effect of antisense oligonucleotide on specific inhibition of mKv1.1 gene expression

The lowering of Kv1.1 mRNA following antisense oligonucleotide administration as an index of Kv1.1 gene

Table 4
Lack of effect of repeated i.c.v. injections of an antisense ODN (aODN) to mKv1.1 gene in the mouse rotarod test

	Number of falls in 30 s			
	Pretest	After s.c. saline injection		
		15 min	30 min	45 min
Vector	4.9 ± 0.6	3.9 ± 0.9	3.0 ± 1.0	2.6 ± 1.1
aODN 2 nmol	5.9 ± 0.4	5.1 ± 0.6	4.0 ± 0.8	3.1 ± 0.8
dODN 2 nmol	5.0 ± 0.8	3.8 ± 0.6	3.2 ± 0.5	3.0 ± 0.5

Mice were i.c.v. injected with vector, antisense ODN (2.0 nmol per injection) or degenerated ODN (dODN) (2.0 nmol per injection) on days 1, 4 and 7. The rotarod test was performed 72 h after the last i.c.v. injection. There were 10 mice per group.

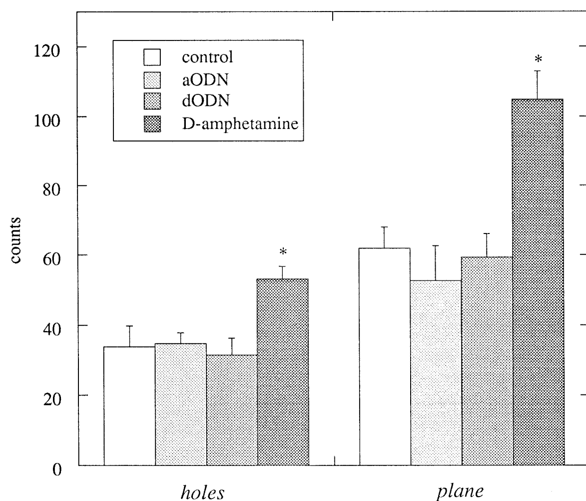


Fig. 6. Lack of effect of repeated i.c.v. injections of an antisense ODN (aODN) to mKv1.1 gene in the mouse hole-board test in comparison with D-amphetamine (1 mg kg^{-1} s.c.) used as reference drug. Mice were injected with vector, antisense ODN (2.0 nmol per injection) or degenerated ODN (dODN) (2.0 nmol per injection) on days 1, 4 and 7. The hole-board test was performed 72 h after the last i.c.v. injection. D-Amphetamine was injected 15 min before the test. Vertical lines give S.E.M.; the number of mice is shown in parentheses. * $P < 0.05$ in comparison with vector-treated mice.

inactivation was quantified by RT-PCR. The quantitative results for Kv1.1 mRNA brain levels following antisense oligonucleotide treatment confirmed that the blockade of clomipramine- and amitriptyline-induced antinociception is associated with the specific Kv1.1 mRNA lowering (Fig. 7).

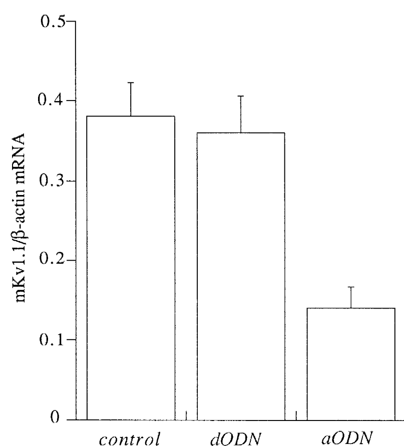


Fig. 7. Quantitative RT-PCR analysis of mKv1.1 mRNA. Following antisense ODN (aODN) or degenerated ODN (dODN) treatment, total RNA was extracted from the brains of behaviourally tested mice. mKv1.1 and β-actin mRNA were submitted to RT-PCR as reported in Section 2. Amplification products were analysed on agarose gel and quantified by densitometry. Within the linearity of PCR, the amount of mKv1.1 mRNA relative to β-actin mRNA was calculated. Data are the means of three determinations. Vertical lines represent S.E.M.

4. Discussion

Repeated i.c.v. administration of an antisense targeted to mKv1.1, a *Shaker*-like K^+ channel, inhibits the analgesia induced by the tricyclic antidepressant drugs clomipramine and amitriptyline. Under our experimental conditions, the antisense oligonucleotide treatment did not modify the gross behaviour of mice. The motor coordination, spontaneous motility and inspection activity of mice pretreated with the antisense oligonucleotide were not impaired in comparison with those of groups treated with degenerated oligonucleotide and vector making the results obtained in the hot-plate test reliable. Moreover, the inhibition of tricyclic antidepressant analgesia by anti-mKv1.1 antisense disappeared 7 days after the last i.c.v. injection, indicating a lack of irreversible damage or toxicity on cerebral structures caused by the antisense oligonucleotide.

Pretreatment with the anti-mKv1.1 antisense did not modify the pain threshold, showing the absence of any hyperalgesic effect. Therefore, the prevention of clomipramine and amitriptyline antinociception cannot be attributable to a direct effect on the pain threshold induced by the antisense oligonucleotide. Furthermore, the degenerated oligonucleotide did not antagonise clomipramine- and amitriptyline-induced antinociception in comparison with naive or saline- and vector-i.c.v. injected mice. This observation ruled out the possibility that the antagonism exerted by antisense oligonucleotide may have resulted from a sequence-independent action on cerebral structures. This hypothesis was further supported by results of a quantitative RT-PCR study demonstrating a reduction in mKv1.1 mRNA levels only on the antisense oligonucleotide-treated group.

The present results not only indicated an important role of mKv1.1 in the mechanism of analgesic action of clomipramine and amitriptyline, but also confirmed the usefulness of antisense strategies for in vivo investigations. Antisense oligonucleotides have proved to be useful pharmacological tools for exploring biological processes in the central nervous system (CNS) both in vitro and in vivo (Chung et al., 1995; Standifer et al., 1994; Rossi et al., 1994; Zhang et al., 1994; Wahlestedt, 1994). Antisense oligonucleotides specifically bind to targeted mRNA, preventing translation and/or mediating mRNA cleavage by RNase H and, thus, down-regulate the synthesis of the encoded protein. Low cell permeability and the marked degradation of natural phosphodiester oligomers are considerable drawbacks in the application of antisense oligonucleotides both in vitro and in vivo. In order to overcome these drawbacks, phosphorothioate-capped phosphorodiester oligonucleotides, a class of oligonucleotide derivatives that has been shown to maintain more stable and effective concentrations in the brain when compared to their unmodified counterpart (Whitesell et al., 1993), were used. Furthermore, both oligonucleotide stability and cell uptake have been enhanced by associating oligonucleotides

with an artificial cationic lipid (DOTAP) used as an intracellular carrier (Capaccioli et al., 1993; Quattrone et al., 1994).

We investigated the involvement of mKv1.1 in the analgesia induced by clomipramine and amitriptyline since mKv1.1 K^+ channels seem to play an important role in regulating neuronal function. The mKv1.1 channel is widely expressed in mouse brain (Adams et al., 1991; Wang et al., 1994) including areas involved in the regulation of the pain threshold. Moreover, mKv1.1 plays a prominent role in the hippocampus where it seems to be involved in the regulation of membrane repolarization and in the duration or amount of neurotransmitter release (Wang et al., 1994).

Little is known about the turnover of the protein Kv1.1 in mouse brain. Results of longer pulse-chase experiments with mouse L-cells indicate that Kv1.1 degradation has a $t_{1/2}$ of approximately 5 h (Deal et al., 1994). However it has to be noted that, in L-cells, cell surface channels are represented by two molecular mass species, 57 and 59 kDa, while the Kv1.1 channel in mouse brain appears as a single 80 kDa species (Wang et al., 1993). It is, therefore, difficult to extend the data obtained in L-cells to the mouse brain because it is possible that this differential processing between the L-cell system and brain has functionally significant consequences.

The administration schedule of antisense oligonucleotide (a single i.c.v. injection every 72 h for a total of 3 injections) was chosen on the basis of in vivo antinociception experiments reported in the literature in which a minimum treatment time of 3–5 days seems necessary to achieve inhibition of neurotransmitter receptor synthesis (Rossi et al., 1994; Chien et al., 1994; Chen et al., 1995; Standifer et al., 1994; Tseng and Collins, 1994; Tseng et al., 1994). Furthermore, the Kv1.1 mRNA levels were reduced 48 h after treatment (data not shown) and a maximal lowering was reached at 72 h. The validity of the treatment schedule used was further supported by the evidence that, after a single intrathecal injection, antisense oligonucleotide accumulates within the spinal cord and remains stable for at least 72 h (Standifer et al., 1995).

The treatment with the antisense oligonucleotide to the mKv1.1 gene prevented the antinociception induced by clomipramine and amitriptyline, indicating the involvement of the *Shaker*-like voltage-gated K^+ channels in the enhancement of the pain threshold produced by the activation of the serotoninergic system. In fact, clomipramine and amitriptyline analgesia implies an enhancement of serotonin levels in the synaptic cleft. The intracellular mechanism of analgesic action of clomipramine and amitriptyline involves the activation of a pertussis toxin-sensitive G-protein, since not only clomipramine (Galeotti et al., 1996) but also amitriptyline analgesia is prevented by the i.c.v. administration of pertussis toxin. Pertussis toxin-sensitive G-proteins represent the most widespread modulatory signalling pathway in neurones (Holz et al., 1986) and are responsible for modulation of ionic conductance and/or lowering of intracellular cyclic AMP levels (Brown and Birnbaumer, 1990; Hepler and Gilman, 1992; Hille, 1994). Physiological evidence suggests that voltage-gated K^+ channels can also be modulated by neurotransmitters and hormones in many tissues. Indeed, protein kinase A modulation of delayed rectifier-type K^+ currents has been shown in the heart and in lymphocytes (Giles et al., 1989; Soliven and Nelson, 1990). More recently an increase in mKv1.1 K^+ channel expression at the levels of RNA, protein and current density in Chinese hamster ovary cells was found as a result of reduced basal protein kinase A activity (Bosma et al., 1993). Serotonin has both excitatory and inhibitory actions in the CNS since it can interact with a large number of 5-HT receptor subtypes coupled to different second messenger systems such as adenylate cyclase, and is able to modulate different ion channels, such as some K^+ channels (Zifa and Fillion, 1992; Lucas and Hen, 1995). Since clomipramine and amitriptyline analgesia is mediated by the serotoninergic system with the involvement of a pertussis toxin-sensitive G-protein, and mKv1.1 function is regulated by protein kinase A levels, the mKv1.1 K^+ channel might be involved in tricyclic antidepressant-induced analgesia as an intracellular effector underlying the activation of a $G_{i/o}$ protein.

In conclusion, the present data demonstrate that mKv1.1 K^+ channels are an important step in the transduction mechanism underlying central antinociception induced by clomipramine and amitriptyline.

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