British Journal of Pharmacology (2004) 142, 381–389
www.nature.com/bjp

Methylamine, but not ammonia, is hypophagic in mouse by interaction with brain Kv1.6 channel subtype

*,[1]Renato Pirisino, [1]Carla Ghelardini, [2]Alessandra Pacini, [1]Nicoletta Galeotti & [1]Laura Raimondi

[1]Department of Preclinical and Clinical Pharmacology, University of Florence, Viale Pieraccini, 6 50134 Florence, Italy and [2]Department of Anatomy, Histology, Forensic Medicine, University of Florence, Viale Morgagni, 85 50134 Florence, Italy

1 Ammonia and methylamine (MET) are endogenous compounds increased during liver and renal failure, Alzheimer's disease, vascular dementia and diabetes, where they alter some neurobehavioural functions probably acting as potassium channel blockers.

2 We have already described that potassium channel blockers including tetraethylammonium (TEA), ammonia and MET are hypophagic in mice. Antisense oligonucleotides (aODNs) against *Shaker-like* Kv1.1 gene abolished the effect of TEA but not of ammonia and MET.

3 The central effects elicited in fasted mice by ammonia and MET were further studied. For MET, an ED_{50} value 71.4 ± 1.8 nmol mouse^{-1} was calculated. The slope of the dose–response curves for these two compounds and the partial hypophagic effect elicited by ammonia indicated a different action mechanism for these amines.

4 The aODNs pretreatments capable of temporarily reducing the expression of all seven known subtypes of *Shaker-like* gene or to inactivate specifically the Kv1.6 subtype abolished the hypophagic effect of MET but not that of ammonia.

5 Reverse transcription–polymerase chain reaction, Western blot and immunohistochemical results indicate that a full expression in the brain of Kv1.6 is required only for the activity of MET, and confirms the different action mechanism of ammonia and MET.
British Journal of Pharmacology (2004) **142,** 381–389. doi:10.1038/sj.bjp.0705740

Keywords: Ammonia; methylamine; mouse; antisense oligodeoxyribonucleotides; Kv1.6 channels; food intake

Abbreviations: $aODN_s$, antisense oligodeoxyribonucleotides; RT–PCR, reverse transcription–polymerase chain reaction; PBS, phosphate-buffered saline; MET, methylamine; TEA, tetraethylammonium

Introduction

Methylamine (MET), a short aliphatic amine present in mammals, derives from the endogenous deamination of adrenaline, sarcosine, creatinine and lecithin, or from food and drink (Zeisel & Da Costa, 1986; Buffoni, 1995). Ammonia (NH_3) shares with MET some metabolic properties in vertebrates also resulting from the endogenous or exogenous catabolism of amino acids and proteins or from the oxidative deamination of different amines (Cooper & Plum, 1987).

Although an increase in the urinary excretion of MET during pregnancy, parturition and muscular exertion has been observed (Dar *et al*., 1985; Precious *et al*., 1988), the general physiological significance of this short aliphatic amine, less investigated than NH_3, is far from being completely clarified.

Experimental and clinical studies point to the important pathological role of NH_3, MET and other basic compounds, such as neurotoxins (Szerb & Butterworth, 1992; Raabe, 1994; Butterworth, 2000; Olde Damink *et al*., 2002). These weak bases increase in hyperammoniaemias during liver or renal diseases (Simenhoff, 1975; Baba *et al*., 1984; Yu & Dyck, 1998) or in Alzheimer's disease and vascular dementia (Seiler, 2002; Yu *et al*., 2003), altering, depending on the tissue levels reached, the metabolism, morphology and excitability of central tissues. In diabetes, the increased endogenous levels of MET are suspected of having a role in central and peripheral vascular degeneration because of the formation, from its oxidative deamination, of the angiotoxic compounds formaldehyde and H_2O_2 (Yu *et al*., 2003).

A common feature of NH_3 and MET is that both compounds induce an exocytotic process in excitable cells mainly through two postulated mechanisms: (i) an intravesicular alkalinization (Seglen, 1983; Mundorf *et al*., 1999) or (ii) a blocking property of different type of potassium currents in the cells (Szerb & Butterworth, 1992; Moroni *et al*., 1998; Hrnjez *et al*., 1999). Consistent with both of these, neurotransmitter release and consequently some behavioural and neurological functions including arousal, learning, pain perception and motor activity could be modified in hyperammonaemic situations (Kuta *et al*., 1983; Szerb & Butterworth, 1992; Aguilar, 2000).

Neurobehavioural investigations have recently shown that different voltage-dependent potassium channel blockers, including NH_3, MET and tetraethylammonium (TEA), reduce the food intake in starved mice by acting as depolarizing compounds in the brain (Ghelardini *et al*., 1997; Banchelli *et al*., 2001; Pirisino *et al*., 2001; Ghelardini *et al*., 2003). In some experiments, an antisense oligodeoxyribonucleotide (aODN) targeting the translation start region of the *Shaker-like* Kv1.1 gene ($aODN_1$) abolished the anorectic effect of TEA, suggesting that functionally active Kv1.1 channel

*Author for correspondence; E-mail: renato.pirisino@unifi.it
Advance online publication: 20 April 2004

subtypes in the brain are required for the activity of this compound. However, the observation that the hypophagic effect of NH_3 and MET was unaffected by this aODN indicated a possible involvement of channel subtypes different from Kv1.1 for the hypophagic effect of these latter compounds (Pirisino *et al*., 2001).

Starting from this hypothesis, because of the aforementioned physiopathological relevance of NH_3 and MET, the present work was undertaken in order to investigate the possibility that these amines perform their hypophagic effect through an interaction with voltage-activated potassium channels different from Kv1.1 subtype. An antisense strategy capable of temporarily knocking down the expression of all seven known subtypes of *Shaker-like* genes was used to achieve this aim.

Methods

Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. In all, 15 mice or five rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization purposes. The animals were fed a standard laboratory diet and tap water *ad libitum*; they were kept at $23 \pm 1^{\circ}C$, with a 12 h light/dark cycle, and light at 0700. All experiments were carried out in accordance with the European Community Council's Directive of 24 November 1986 (86/609/EEC) relative to experimental animal care. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Evaluation of food consumption

The mice did not have access to food for 12 h, but water was available *ad libitum*. A weighed amount of food (standard laboratory pellets) was given, and the amount consumed (evaluated as the difference between the original amount and the food left in the cage, including spillage) was measured after 15, 30, 45 and 60 min, in order to evaluate the time course of each treatment, after the intracerebroventricular (i.c.v.) administration of saline or drug solutions, with an accuracy of 0.1 g. An arbitrary cutoff time of 60 min was adopted, and the total amount of food consumed was expressed in mg mouse h^{-1}. A constant decrease in the food intake was observed at every interval, which indicated that the hypophagic effect of the treatments did not disappear within 60 min. The cutoff time used in our experiments (60 min) corresponded to the maximum food intake observable in control animals: after 60 min, the amount of food intake drastically decreased in the control group. The food intake was measured in animals housed individually, for acclimatization, in the cage 12 h before inducing the food deprivation. The experiment was then performed in the same cage.

Drug administration by i.c.v. route

The i.c.v. administration took place under ether anaesthesia with isotonic saline used as solvent, according to the method described by Haley & McCormick (1957) without the assistance of a stereotaxic apparatus. During anaesthesia, the mice were grasped firmly by the loose skin behind the head. The effects produced by ether anaesthesia and by i.c.v. injection were determined in different groups of mice: naive animals, animals that received anaesthesia without receiving an i.c.v. injection and animals that received an i.c.v. injection of saline. No difference in the food intake among groups was observed. A hypodermic needle (0.4 mm external diameter) 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 a 5 μl solution was then administered. The injection site was 1 mm to the right or left of the midpoint on a line drawn through to the anterior base of the ears. Injections were performed into the right or left ventricle. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 5 μl of diluted 1 : 10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated, with 95% of the injections being correct. Owing to this high percentage of correct injections, no animal was excluded.

Before i.c.v. administration of the compounds used, it was assessed that the pH values of the nM compound solutions, ranging from 7.2 to 6.7, did not vary significantly from those of the saline ($pH = 6.8 \pm 0.4$).

Design of aODNs

An anti-mouse aODN against all the Kcna isoforms, named anti-Kv1–7 aODN ($aODN_{1-7}$), was designed comparing the sequences of the known mouse Kcna coding genes (Kcna1, Kcna2, Kcna3, Kcna4, Kcna5, Kcna6 and Kcna7) and choosing a common and conserved region with a 92% homology. The sequence of the $aODN_{1-7}$ was: 5′-TGG CGG GAG AGC TTG AAG AT-3′. In order to knockdown single Kcna isoforms, we designed seven specific anti-mouse Kcna aODNs ($aODN_1$, $aODN_2$, $aODN_3$, $aODN_4$, $aODN_5$, $aODN_6$ and $aODN_7$), the sequences of which are shown in Table 1. Moreover, in consideration of the described sequence-independent, nonantisense effects of ODNs described, we designed one type of control: a 20-mer fully degenerated ODN (dODN), 5′-NNN NNN NNN NNN NNN NNN NN-3′ (where N is G, or C, or A, or T). The aODN and the control dODN were phosphorothioate protected by a 5′- and 3′-end double substitution (phosphorothioate residues are emphasized), synthesized on a 10-μmol scale and purified by HPLC. ODN_s were vehiculated intracellular by cationic lipid DOTAP

Table 1 Anti-Kcna1, Kcna2, Kcna3, Kcna4, Kcna5, Kcna6 and Kcna7 ODN sequences

$aODN_s$	*$aODN_s$ sequences*	*GenBank Accession Numbers*
$aODN_{1-7}$	TGG CGG GAG AGC TTG AAG AT	
$aODN_1$	GCATTCTCCCCCGACATCAC	NM_010595
$aODN_2$	ACTGGGTCTCCGGTAGCCAC	NM_008417
$aODN_3$	CGCCGCCACTGCCAC	NM_008418
$aODN_4$	GTACGAACACCCATCCCCAT	NM_021275
$aODN_5$	CTCCTCATCCTCAGCA	AF302768
$aODN_6$	CCCGGCGCCGCCAGCGTCAG	NM_013568
$aODN_7$	GCAGCCACCCCAGTCGGGTG	AF032099

(13 μM) Sigma Chemical Company (St. Louis, MO, U.S.A.) to enhance both uptake and stability (Whitesell *et al*., 1993).

Semiquantitative reverse transcription–polymerase chain reaction (RT—PCR)

For the quantification of Kv1.6. mRNA levels, total RNA was extracted by Tri Reagent Sigma Chemical Company (St. Louis, MO, U.S.A.) according to the manufacturer's protocol. Semiquantitative determination of Kv1.6 mRNA levels was carried out by an internal standard-based RT–PCR assay with serial dilution of total RNA and using β-actin as reference gene, as described previously (Pacini *et al*., 1999). Total RNA (200 ng) were reverse transcribed and amplified by the Super-Script One-Step RT–PCR System (Invitrogen, Europe). A 219 bp segment of the murine Kv1.6 cDNA sequence was targeted with upstream primer 5′-GGC TTC CTT CTC ATG CTC AC-3′, bases 2559–2578 and downstream primer 5′-GTG ACT GAT GGG CAT GAT TG-3′, bases 2759–2778, while a 326 bp segment of the murine β-actin sequence (GenBank™ Accession Number NM_007393) was amplified with upstream primer 5′-GCG GGA AAT CGT GCG TGA CAT T-3′, bases 2106–2127, and downstream primer 5′-GAT GGA GTT GAA GGT AGT TTC GTG-3′, bases 2409–2432. The RT–PCR profile was: one cycle of 55°C for 30 min and 94°C for 2 min (cDNA synthesis and predenaturation) followed by PCR amplification performing 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 1 min. Amplification products were run on a 2% agarose gel and the ethidium bromide-stained bands were quantified by densitometric analysis. Within the linear range of amplification, at least three values of Kv1.6 amplification products were normalized to the starting total RNA volumes and referred to the corresponding β-actin values.

Western blotting

Mouse brains ($\sim$0.2 g) of control ($dODN_6$) and antisense-treated ($aODN_6$) mice were homogenized on ice in 1 ml of lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 0.1 mg ml^{-1} protease inhibitor cocktail (Complete, Roche, Milan, Italy). Homogenates were then centrifuged at 13,000 $\times$ *g* for 15 min, and the resulting supernatants underwent determination of protein concentration by the bicinchoninic acid reagent Sigma Chemical Company (St. Louis, MO, U.S.A.). Lysates containing 30 μg of proteins were subjected to SDS–PAGE on a 10% polyacrylamide gel. Western blot analyses were performed using anti-Kv1.6 polyclonal antibody (Santa Cruz Biotechnology, Europe), followed by peroxidase-conjugated secondary antibody Sigma Chemical Company (St. Louis, MO, U.S.A.) at a dilution of 1 : 2000. The blots were developed using Opti-4CN kit (BioRad, Europe).

Immunohistochemistry

For immunochemical experiments, control mice ($dODN_6$) were overdosed with 5% chloral hydrate by means of intraperitoneal administration and were perfused transcardially with 50 ml of cold phosphate-buffered saline (PBS, 0.02 M, pH 7.4), followed by 100 ml of 4% paraformaldehyde in PBS. Brains were removed immediately and placed in 4% paraformaldehyde at 4°C for 24 h; they were cryoprotected in a series of cold sucrose solutions of increasing concentration. Frozen brains were cut at 8 μm in the coronal plane and were incubated in a 1 : 200 dilution of primary antibody (anti-Kv1.6, Santa Cruz Biotechnology, Europe) overnight at 4°C, followed by a 30 min wash in PBS. The sections were then immersed for 1 h in the peroxidase-conjugated rabbit anti-goat immunoglobulin G diluted at 1 : 500 in PBS. Next, the sections were rinsed for 30 min in PBS, followed by a 15-min incubation in NovaRED substrate (VECTOR NovaRED substrate kit, Vector, Peterborough, U.K.). Sections were counterstained with toluidine blue and then mounted on slides. Control sections were treated as described above, but omitting the primary antibody.

Reagents and drugs

Ammonium acetate, MET HCl and TEA chloride used in the food intake experiments were purchased from the Sigma-Aldrich Chemical Company (Milan, Italy). The compounds were dissolved in isotonic (NaCl 0.9%) saline. Drug concentrations were prepared in such a way that the necessary dose could be administered by i.c.v. injection in a volume of 5 μl $mouse^{-1}$. The ODNs used for the antisense strategy were from Genosys (The Woodlands, U.S.A.). DOTAP was from Boheringer-Mannheim (Mannheim, Germany). Antisense and degenerated ODNs were dissolved in the vector (DOTAP) at least 30 min before injection.

Statistical analysis

All experimental results are given as the mean $\pm$ s.e.m. An analysis of variance was used to verify the significance between two means of the behavioural results, and was followed by Fisher's protected least significant difference procedure for *post hoc* comparison. Data were analysed using the Stat View software for Macintosh (1992). *P*-values of less than 0.05 were considered significant.

Results

Food intake experiments

Figure 1 shows a comparison from dose–response experiments for the hypophagic effect of i.c.v.-injected MET and NH_3 in

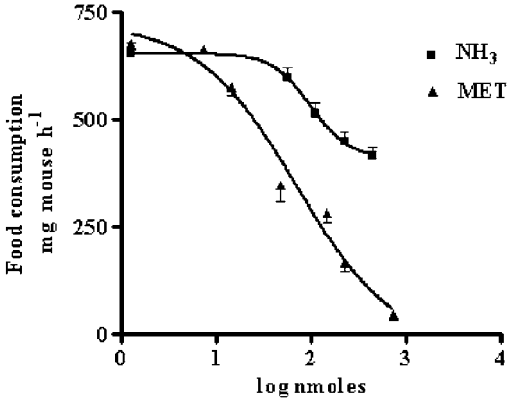

Figure 1 Dose-related reduction of food intake in 12 h starved mice after the i.c.v. injection of MET or NH_3, as measured 60 min after food readministration. Each point represents the mean $\pm$ s.e.m. of at least 10 mice.

12-h starved mice. Expressed as the dose of compound required to produce a 50% reduction of feeding, an ED_{50} value (71.4 ± 1.8 nmol mouse^{-1}) was calculated only for MET, since highest reduction induced by NH_3 was about 30% of the total food consumption in control mice.

Effect of aODN$_1$ and aODN$_{1-7}$ pretreatments

In Table 2, the hypophagic effects of equivalent doses (about three times higher than MET ED_{50}; 226 nmol) of MET and NH_3, injected i.c.v. in 20 nmol aODN$_{1-7}$-pretreated mice, are reported. As detailed in the Methods section, this aODN was specifically designed to abolish contemporaneously the expression of all seven known subtypes of *Shaker-like* Kv1 channels. In Table 2, the effect induced by repeated administration of this aODN on the hypophagic effect of NH_3, MET and TEA is also compared with the effect elicited by 3 nmol aODN$_1$ pretreatment. A dose of 20 nmol of aODN$_{1-7}$ was selected for these experiments on the basis of preliminary results, which showed that this quantity reduced the hypophagic response of 30 nmol i.c.v. TEA (taken as a reference compound) of an amount similar to the one previously observed when pretreatments with 3 nmol of aODN$_1$ were used (Pirisino *et al*., 2001). Moreover, the administration schedule of aODN$_{1-7}$ (a single i.c.v. injection on days 1, 4 and 7 before the administration of compounds) was the same as the one previously used in experiments with aODN$_1$ (Galeotti *et al*., 1997a, b; Ghelardini *et al*., 1997). In these, by means of quantitative RT–PCR analysis, it was observed that this treatment significantly reduced (by more than 60%) the expression of the Kv1.1 channel subtype in the mouse brain.

As shown in Table 2, the hypophagic effect of both MET or NH_3 was unaffected by pretreatments with aODN$_1$. However, in mice pretreated with 20 nmol of the aODN$_{1-7}$, a significant inhibition was observed only for the hypophagic effect of MET, despite the fact that the recovery of the food consumption was incomplete when compared with the food intake observed in controls, saline-injected, dODN$_{1-7}$-pretreated mice. In contrast, the hypophagic responses of TEA were almost completely reversed in mice pretreated with both aODN$_1$ or aODN$_{1-7}$.

The ability of aODN$_{1-7}$ in counteracting the hypophagic effect of MET was also evaluated by increasing the dosage of this aODN. In Figure 2, a progressive reduction of the MET effect is shown with maximum effect between 20 and 40 nmol of aODN$_{1-7}$. In the experiments reported in Table 2, as well as in Figure 2, the degenerated ODN (dODN$_1$ or dODN$_{1-7}$) were both unable to modify significantly the food consumption of saline-injected animals.

The motor coordination in mice after different pharmacological treatments was also evaluated by the Hole-board test, as described previously (Ghelardini *et al*., 2003), starting 15 min after the i.c.v. injection of the test compounds or 48 h after the last ODN injection. As all the compounds, at the highest active doses employed in the present study, did not cause any detectable modification in mouse gross behaviour (motor coordination, spontaneous motility or inspection activity) in comparison with control groups (data not shown), excluding that the results obtained were due to an altered viability of animals, such experiments are not reported for brevity in the text.

Effect of aODNs relative to each single Kv1 subtype on MET activity

The results obtained in mice pretreated with aODN$_1$ or aODN$_{1-7}$ indicated that one or more subtypes of *Shaker-like* channels ranging from Kv1.2 and Kv1.7 were probably the molecular targets for the hypophagic activity of MET. To

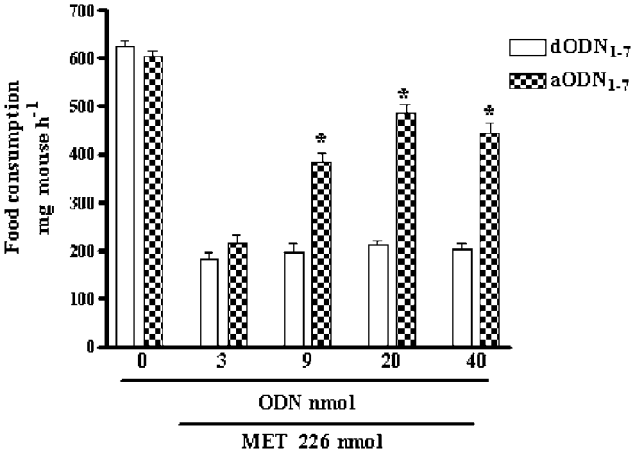

Figure 2 Effect of increasing concentrations (i.c.v. injection at days 1, 4 and 7) of aODN$_{1-7}$ or dODN$_{1-7}$ on MET-induced food consumption in 12 h starved mice, as measured 60 min after food readministration. Each point represents the mean ± s.e.m. of at least 10 mice. *$P<0.01$ in comparison with dODN$_{1-7}$-pretreated mice.

Table 2 Reduction of the hypophagic effect of NH_3-, MET- and TEA in ODNs-pretreated 12 h fasted mice

Compound, 226 nmol/*5 μl i.c.v.*	*Food consumption* (mg mouse h^{-1})							
	dODN$_1$	%[a]	*aODN$_1$*	%[a]	*dODN$_{1-7}$*	%[a]	*aODN$_{1-7}$*	%[a]
Saline	653 ± 26	—	697 ± 20	—	623 ± 12	—	604 ± 11	—
NH_3	449 ± 23	23	429 ± 25	38	414 ± 23	33	451 ± 18	25
MET	154 ± 21	76	131 ± 27	81	211 ± 10	66	[b]487 ± 18	19
[c]TEA	381 ± 16	49	[b]674 ± 25	3	313 ± 70	50	[b]583 ± 17	3

All tested compounds significantly reduced the food intake ($P< 0.01$) in dODN$_s$-treated animals compared with control mice (saline injected). In all, 3 nmol of a/dODN$_1$ and 20 nmol of a/dODN$_{1-7}$ were injected i.c.v. in 5 μl of DOTAP solution.
[a]Percentage reduction vs saline, ODN$_s$-pretreated controls. Each value represents the mean ± s.e. mean of at least 10 animals.
[b]Significant ($P<0.01$) in comparison with the anorectic effect of the compound in dODN$_s$-pretreated animals.
[c]TEA^+ 30 nmol i.c.v.

confirm this hypothesis, the effect of MET was evaluated in mice pretreated with $aODN_s$ specifically designed to block the expression of each single *Shaker-like* channel subtype in the mouse brain (see Table 1 for reference). As a result (Figure 3), a significant inhibition of the hypophagic effect of MET was obtained only in mice receiving the aODN towards Kv1.6 subtype, in accordance with the general scheme of aODN administration reported in Methods section. In Figure 3, the absence of any interference on food intake in control, starved mice pretreated with 9 nmol of $aODN_6$ or $dODN_6$ strongly supported the view that these ODNs where devoid '*per se*' of any type of central activity at the dosage employed. Similar results were obtained in control mice pretreated with ODNs specific for the other channel subtypes (data not shown).

A pretreatment with 9 nmol of $aODN_6$ was used in these experiments, because this was preliminarily assessed as the smaller dose of $aODN_6$ almost equipotent to 20 nmol of $aODN_{1-7}$ in reducing the MET-induced hypophagic response in mice.

Time dependence of the $aODN_6$ on MET effects

The $aODN_6$ pretreatment (9 nmol i.c.v. per mouse) prevented the hypophagic response of MET, and this effect was detected 24 h after the end of the $aODN_6$ pretreatment. In contrast, 7 days after the last $aODN_6$ injection, the effect of the $aODN_6$ disappeared and the same animals were once again sensitive to the anorectic effect of MET (Figure 4).

Effect of $aODN_6$ on mKvl.6 gene expression

The lowering of Kv1.6 mRNA after aODN administration, as an index of Kv1.6 gene expression inactivation, was quantified by RT–PCR. Before quantification, RT products were preliminarily tested for possible genomic DNA contamination. For this purpose, segments of 200 bp for mouse Kv1.6 cDNA and of 326 bp for β-actin cDNA, visualized by agarose-gel electrophoresis (Figure 5a), showed bands of an expected length and the absence (data not shown) of any contamination in the negative controls. Quantitative results of Kv1.6 and β-actin mRNA brain levels after $aODN_6$ (9 nmol mouse^{-1} i.c.v.) pretreatment confirmed that, in starved mice, the MET-induced hypophagic effect were actually related by the specific inhibition of Kv1.6 gene expression. These results show that the ratio of Kv1.6 mRNA over β-actin mRNA was sharply lowered (about 70% reduction) in $aODN_6$-treated mice, as compared with $dODN_6$-treated ones. The decrease in specific mRNA was in good correlation with a significant reduction (about 50% reduction) of the phenotypic expression of the 80 kDa Kv1.6 protein level (Figure 5b) in brain homogenates obtained from aODN-treated mice as well as with the reduction of hypophagic response of MET (Figures 3 and 4). Some experiments were also conducted on brain tissues of mice pretreated with $aODN_s$ designed to be selective for every other *Shaker-like* channel subtype. These $aODN_s$ induced a lowering in specific mRNA levels to an extent similar to that observed in $aODN_6$-treated mice. However, because these $aODN_s$ were without any effect in reducing the hypophagic response of MET, no further investigation was made of their properties.

Effect of $aODN_6$ on hypothalamic Kv1.6 protein expression

To evaluate whether the reduction of Kv1.6 protein observed in whole brain mouse homogenates corresponded to a similar reduction in specific brain areas of $aODN_6$-treated mice, we performed some preliminary, immunohistochemical, experiments on brain sections of the same mice used to assess food consumption. In $dODN_6$-pretreated mice, we localized a high expression of Kv1.6 in different brain areas of the mouse. Hypothalamic neurons, for example, showed strong Kv1.6 immunoreactivity in the lateroanterior hypothalamic nucleus (LAH), with a relatively marked staining in the plasmatic membrane of cell bodies (Figure 5c, left panel). Kv1.6

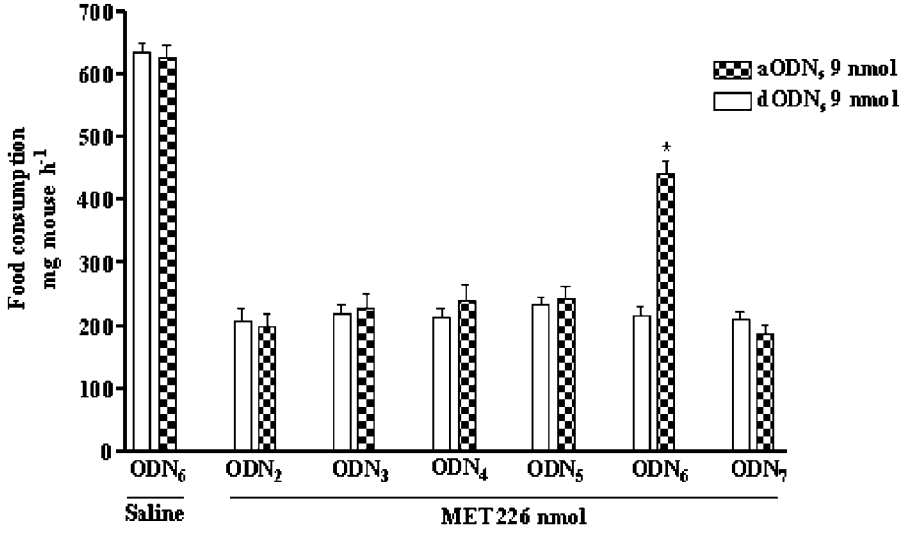

Figure 3 Effect of $aODN_2$, $aODN_3$, $aODN_4$, $aODN_5$, $aODN_6$, $aODN_7$ and related $dODN_s$ (controls) (9 nmol per single i.c.v. injection at days 1, 4 and 7) on MET-induced food consumption in 12 h fasted mice, as measured 60 min after food readministration. Each point represents the mean $\pm$ s.e.m. of at least 10 mice. $^*P<0.01$ in comparison with $dODN_6$-pretreated mice.

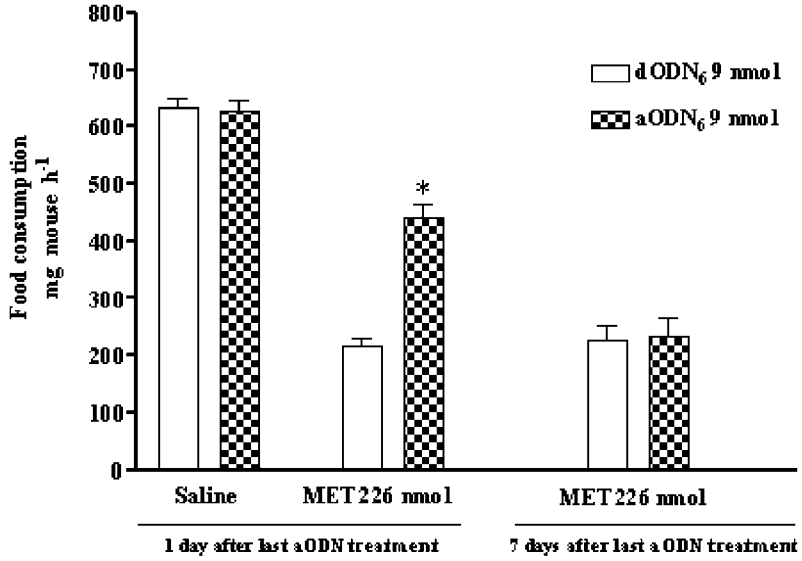

Figure 4 Recovery of the hypophagic response of i.c.v.-administered MET (226 nmol) in $aODN_6$- or $dODN_6$- (controls) (9 nmol per single i.c.v. injection at days 1, 4 and 7) pretreated mice. At 7 days after the last aODN injection, the inhibitory effect of the $aODN_6$ was completely lost. $^*P<0.01$ in comparison with $dODN_6$-pretreated mice taken as controls. Each point represents the mean ± s.e.m. of at least 10 mice.

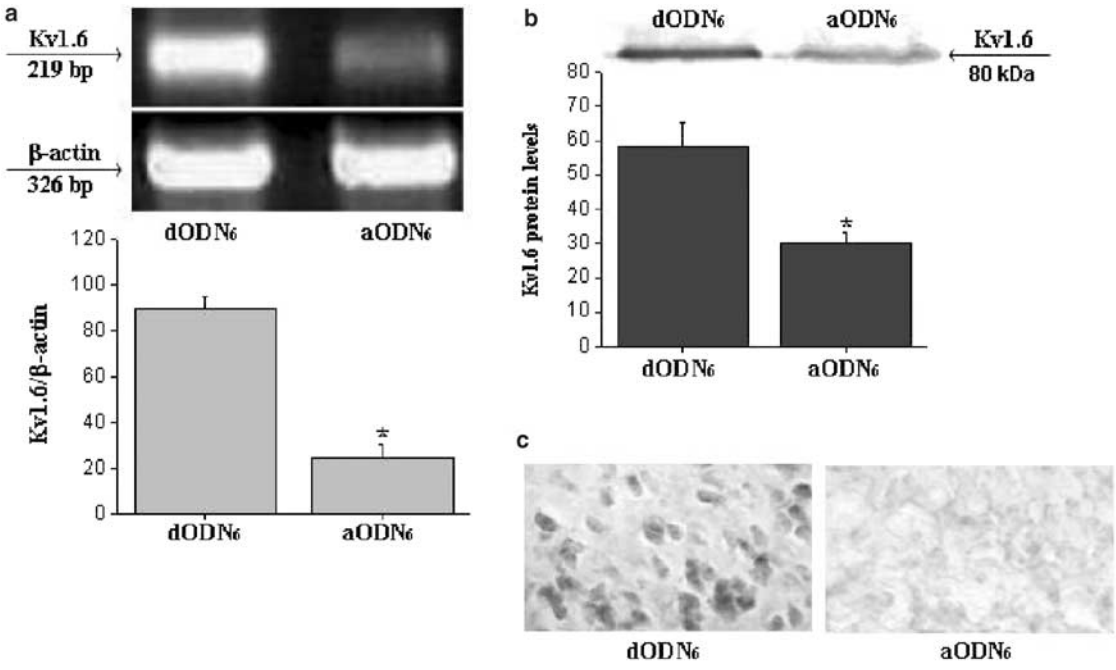

Figure 5 (a) Lowering of Kv1.6 over β-actin mRNA levels in brain homogenates as detected by RT–PCR. (b) Reduction of phenotypic expression of the 80 kDa Kv1.6 protein level in brain homogenates obtained from $aODN_6$-treated mice. (c) Left panel, Kv1.6 immunoreactivity in the LAH of hypothalamic neurons of $dODN_6$- (controls) pretreated mice; evidence for a significant reduction of immunostaining in brain sections from $aODN_6$-pretreated mice (right panel).

immunoreactivity was not observed in sections obtained from $aODN_6$-pretreated animals (Figure 5c, right panel).

Discussion

MET and NH_3, two known endogenous weak bases, when injected i.c.v., dose-dependently reduced food intake in mice. An analysis of the sigmoid curves obtained with these two compounds indicate that MET is more active than NH_3 for this effect. These results agree with previous observations (Pirisino *et al*., 2001), which showed that single doses of these compounds were differently potent in eliciting hypophagic effects when injected i.c.v. in mice starved for 12 h. Moreover, if we look at the slope of the dose–response curves and consider the maximum

effects elicited, it is reasonable to conclude that these two amines work with a probably different mechanism of action.

NH_3 and MET, and other basic compounds, are considered of importance in hepatic or renal insufficiency and in central neurodegenerative disease including Alzheimer's disease and vascular dementia. However, despite the fact that endogenous levels of MET are often increased in those pathological conditions in which NH_3 brain elevations are also seen (Yu *et al*., 2003), minor attention has been paid up, until now, to the specific mechanism of action of this amine, which is often considered, due to its chemicophysical properties, to be similar to that of NH_3.

Alteration of axonal conductance (Raabe, 1989), ionic pumps inhibition (Lux & Neher, 1970) and modification of central 5-HT or dopamine turnover has been described for NH_3 (Szerb & Butterworth, 1992). Such changes are considered to be responsible for alterations to several behavioural functions in experimental animals and ureamic patients (Kuta *et al*., 1983; Szerb & Butterworth, 1992). GABAergic or glutammatergic transmission is also modified in hyperammonaemic status so that, depending on the brain levels, both neuroexcitation and seizures or lethargy, confusion, global CNS depression and coma could develop (Basile, 2002; Zielinska *et al*., 2002). For MET, only indirect evidence are obtained in mice pretreated with MAO A or MAO B inhibitors, which indicates that this compound acts as a central anorexigenic without any apparent interference from the release of monoaminergic mediators (Pirisino *et al*., 2001).

NH_3 and MET are both recognized as acting as exocytotic agents in excitable cells. It has been considered that, because of their liposolubility, these compounds quickly pass in unprotonated form through the plasmatic membrane of the neurons, disrupting – by an elevation of the intragranular pH – the association of neurotransmitters with Ca^{2+}, ATP and vesicular proteins (Seglen, 1983; Mundorf *et al*., 1999). On the other hand, the pK_a values of NH_3 and MET (9.2 and 10.6, respectively) indicate that, under physiological pH, 98–99% of these amines are protonated so that, in this form, they may also enter the cells through ionic channels. It has been shown that, among a series of amines of increasing molecular weight, the protonated form of NH_3 and MET have the highest probability of occupying the channel site for K^+, reducing the conductance of this ion with depolarizing effects. This is in accordance with other results which show that NH_3 and MET, in their protonated form, are able to block different types of potassium currents in the cells (Szerb & Butterworth, 1992; Moroni *et al*., 1998; Hrnjez *et al*., 1999).

Potassium channels represent the largest family of the ion channels having an important role in cellular excitability, regulating neurotransmitter release and other cellular functions. Membrane depolarization open voltage-gated potassium channels conducting outward currents, which in turn leads to repolarization of the membrane. A subfamily of the voltage-operated potassium channels, widely distributed in brain tissue, is related to *Shaker-like* genes encoding for seven members (Kv1.1–Kv1.7) of these channels (Kashuba *et al*., 2001; Chung *et al*., 2001).

The primary purpose of this study was to evaluate the possibility that the hypophagic effects of NH_3 and MET might be due to an interaction of a *Shaker-like* channel subtypes different from Kv1.1. The present investigations confirm some previously reported results (Pirisino *et al*., 2001), showing that neither NH_3- nor MET-induced hypophagic effects are influenced by aODN anti-Kv1.1 channels. When the animals were pretreated with $aODN_{1-7}$, a different pattern of activity resulted. The inhibition of the hypophagic effect of TEA, which was assessed as being sensitive to $aODN_1$, was still observed. However, a significant reversal of the MET-induced effects was seen, suggesting that $aODN_{1-7}$ have reduced the expression of *Shaker-like* channel subtypes different from Kv1.1 ones that probably represent an important molecular target of MET.

Dose–response experiments indicate that $aODN_{1-7}$ maximally inhibited the MET-induced hypophagic effects at concentrations seven times higher than those required by $aODN_1$ for its effects (20 vs 3 nmol, respectively). As reported in the Methods section, the $aODN_{1-7}$ was designed to inactivate contemporaneously the expression of seven different channel subtypes by choosing a common and conserved mRNA region with a 92% homology. By widening the pattern of activity, this procedure could have reduced the specificity of $aODN_{1-7}$ for an individual channel subtype, leading to an increase in the concentrations required to inhibit a single channel expression. On the other hand, pretreatments with $aODN_{1-7}$ were still ineffective in reducing the hypophagic effect of NH_3 in starved mice, further confirming that MET and NH_3 act as hypophagic compounds but with a completely different action mechanism. These observations may provide an explanation for the partial hypophagic response of NH_3 as compared to the total abolition of this response induced by MET. It is reasonable to hypothesize that the release of neuromediators with different hypophagic potency could be differently modulated by NH_3 or MET, as a consequence of the interaction with their respective molecular targets.

To clarify which subtype of the *Shaker-like* potassium channels was responsible for the hypophagic effect of MET, this compound was administered to mice pretreated with $aODN_s$ designed to block the expression of single channel subtypes. Results indicate that the hypophagic effect of MET was significantly reduced only in mice pretreated with the anti-Kv1.6 aODN ($aODN_6$). The reduction in mRNA levels, together with a significant inhibition of specific Kv1.6 protein in brain homogenates of mice used for food intake experiments, confirm that, differently to NH_3, MET require the full expression of Kv1.6 channel subtype in the membrane for its activity. Interestingly, as demonstrated by immunohistochemical analysis, the $aODN_6$ particularly downregulated Kv1.6 the expression in the LAH. As lateral hypothalamus displays high receptor density for different monoaminergic as well as nonmonoaminergic endogenous neuromediators known to participate centrally in the regulation of the food intake (Inui, 2000), it is possible to hypothesize that MET could act as hypophagic mainly in this brain area.

Considering the apparent difference in the action mechanism of NH_3 and MET, it is possible that NH_3, because of its low molecular size and high liposolubility, freely accumulate into neurons by passive diffusion as well as through ionic channels. The intragranular alkalinization and/

or an unselective potassium channel blocking activity should be equally involved in the central effect of this amine. On the contrary, the ammonium analogue MET, at least at the doses employed in this work, could be regarded as a selective blocker of neuronal *Shaker-like* Kv1.6 channel subtype able to modulate, in such way, the release of some hypophagic neuromediators. This view seems to be in some agreement with the electrophysiological results of Moroni *et al*. (1998) and of Hrnjez *et al*. (1999), which show that NH_3, MET and other alkyl amines compete with different selectivity for potassium currents in different cellular experimental models.

Conclusions

To conclude, we have demonstrated for the first time that MET, an endogenous basic compound of physiopathological interest, differently to NH_3, induces its central hypophagic effects by acting on Kv1.6. potassium channels subtypes. These observations may provide more insight into the role of this weak base in pathological conditions in which an increase in the plasma and brain levels of this amine is found.

The financial support for this research was obtained from 2001 Italian Grant of MIUR.

References

AGUILAR, M.A. (2000). Chronic moderate hyperammoniemia impairs active and passive avoidance behavior and conditional discrimination learning in rats. *Exp. Neurol.,* **161,** 704–713.

BABA, S., WATANABE, Y., GEJYO, F. & ARAKWA, M. (1984). High performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure. *Clin. Chim. Acta,* **136,** 49–56.

BANCHELLI, G., GHELARDINI, C., RAIMONDI, L., GALEOTTI, N. & PIRISINO, R. (2001). Selective inhibition of amine oxidases differently potentiate the hypophagic effect of benzylamine in mice. *Eur. J. Pharmacol.,* **413,** 91–99.

BASILE, A.S. (2002). Direct and indirect enhancement of GABAergic neurotransmission by ammonia: implications for the pathogenesis of hyperammonemic syndromes. *Neurochem. Int.,* **41,** 115–122.

BUFFONI, F. (1995). Semicarbazide-sensitive amine oxidases: some biochemical properties and general considerations. *Prog. Brain Res.,* **106,** 323–331.

BUTTERWORTH, R.F. (2000). Complications of cirrhosis III. Hepatic encephalopathy. *J. Hepatol.,* **32,** 171–180.

CHUNG, Y.H., CHUNG-MIN, S., KIM, M.J., LEE, B.K. & CHA, C.I. (2001). Istochemical study on the distribution of six members of the Kv1 channel subunits in the rat cerebellum. *Brain Res.,* **895,** 173–177.

COOPER, A.J.L. & PLUM, F. (1987). Biochemistry and physiology of brain ammonia. *Physiol. Rev.,* **67,** 440–519.

DAR, M.S., MORSELLI, P.L. & BOWMAN, E.R. (1985). The enzymatic system involved in the mammalian metabolism of methylamine. *Gen. Pharmacol.,* **16,** 615–620.

GALEOTTI, N., GHELARDINI, C., CAPACCIOLI, S., QUATTRONE, A., NICOLIN, A. & BARTOLINI, A. (1997a). Blockade of clomipramine and amitriptyline analgesia by an antisense oligonucleotide to mKv1.1, a mouse Shaker-like K^+ channel. *Eur. J. Pharmacol.,* **330,** 15–25.

GALEOTTI, N., GHELARDINI, C., PAPUCCI, L., CAPACCIOLI, S., QUATTRONE, A. & BARTOLINI, A. (1997b). An antisense oligonucleotide on the mouse Shaker-like potassium channel Kv1.1 gene prevents antinociception induced by morphine and baclofen. *J. Pharmacol. Exp. Ther.,* **281,** 941—949.

GHELARDINI, C., GALEOTTI, N., PECORI, V.A., CAPACCIOLI, S., QUATTRONE, A. & BARTOLINI, A. (1997). Effect of K^+ channel modulation on mouse feeding behaviour. *Eur. J. Pharmacol.,* **329,** 1–8.

GHELARDINI, C., QUATTRONE, A., GALEOTTI, N., LIVI, S., BANCHELLI, G., RAIMONDI, L. & PIRISINO, R. (2003). Antisense knockdown of the Shaker-like Kv1.1 gene abolishes the central stimulatory effects of amphetamines in mice and rats. *Neuropsychopharmacology,* **28,** 1096–1105.

HALEY, T.J. & MCCORMICK, W.G. (1957). Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. *Br. J. Pharmacol. Chemother.,* **12,** 12–15.

HRNJEZ, B.J., SONG, J.C., PRASAD, M., MAYOL, J.M. & MATTHEWS, J.B. (1999). Ammonia blockade of intestinal epithelial K^+ conductance. *Am. J. Physiol.,* **277,** G521–G532.

INUI, A. (2000). Transgenic approach to the study of body weight regulation. *Pharmacol. Rev.,* **52,** 35–61.

KASHUBA, V.I., KVASHA, S.M., PROTOPOPOV, A.I., GIZATULLIN, R.Z., RYNDITCHE, A.V., WAHLESTEDT, C., WASSERMAN, W.W. & ZABAROVSKY, E.R. (2001). Initial isolation and analysis of the human Kvl.7 (KCNA7) gene, a member of the voltage-gated potassium channel gene family. *Gene,* **268,** 215–222.

KUTA, C.C., MAICKEL, R.P. & BOROWITZ, J.L. (1983). Modification of drug action by hyperamoniemia. *J. Pharmacol. Expt. Ther.,* **229,** 85–90.

LUX, H.D. & NEHER, E. (1970). The action of ammonium on postsynaptic inhibition of cat spinal motorneurones. *Exp. Brain Res.,* **11,** 431–447.

MORONI, A., BARDELLA, L. & THIEL, G. (1998). The impermeantion methylammonium blocks K^+ and NH_4^+ currents thorough KAT1 channel differently: evidence for ion interaction in channel permeation. *J. Membr. Biol.,* **163,** 25–35.

MUNDORF, M.L., HOCHSTETLER, S.E. & WIGHTMAN, R.M. (1999). Amine weak bases disrupt vesicular storage and promote exocytosis in chromaffin cells. *J. Neurochem.,* **73,** 2397–2405.

OLDE DAMINK, S.W.M., DEUTZ, N.E.P., DEJONG, C.H.C., SOETERS, P.B. & JALAN, R. (2002). Interorgan ammonia metabolism in liver failure. *Neurochem. Int.,* **41,** 177–188.

PACINI, A., QUATTRONE, A., DENEGRI, M., FIORILLO, C., NEDIANI, C., RAMON, Y., CAJAL, S. & NASSI, P. (1999). Transcriptional downregulation of PARP gene expression by E1A binding to pRb proteins protects murine keratinocyted from radiation-induced apoptosis. *J. Biol. Chem.,* **274,** 35107–35112.

PIRISINO, R., GHELARDINI, C., BANCHELLI, G., GALEOTTI, N. & RAIMONDI, L. (2001). Methylamine and benzylamine induced hypophagia in mice: Modulation by semicarbazide-sensitive benzylamine oxidase inhibitors and aODN towards Kv1.1 channels. *Br. J. Pharmacol.,* **134,** 880–886.

PRECIOUS, E., GUNN, C.E. & LYLES, G.A. (1988). Deamination of methylamine by semicarbazide-sensitive amine oxidase in human umbilical artery and rat aorta. *Biochem. Pharmacol.,* **37,** 707–713.

RAABE, W. (1989). Neurophysiology of ammonia intoxication. In: *Hepatic Encephalopathy: Pathophysiology and Treatment,* ed. Butterworth, R. & Pomier-Layrargues, G. pp. 49–77. Clifton, NJ: Humana Press.

RAABE, W. (1994). Effects of hyperammoniemia on neuronal function: NH_4^+, IPSP and Cl^- extrusion. In: *Cirrhosis, Hyperammoniemia and Hepatic Encephalopaty,* ed. Grisolia, F. & Felipo, V. pp. 71–82. New York: Plenum Press.

SEGLEN, P.O. (1983). Inhibitors of lysosomal function. *Methods Enzymol.,* **96,** 737–764.

SEILER, N. (2002). Ammonia and Alzaimer's disease. *Neurochem. Int.,* **41,** 189–207.

SZERB, C.J. & BUTTERWORTH, R.F. (1992). Effects of ammonium ions on synaptic transmission in the central nervous system. *Progr. Neurobiol.,* **39,** 135–152.

SIMENHOFF, M.L. (1975). Metabolism and toxicity of aliphatic amines. *Kidney Int.,* **7,** S314–S317.

WHITESELL, L., GESELOWITZ, D., CHAVANY, C., FAHMY, B., WALBRIDGE, S., ALGER, J.R. & NECKERS, L.M. (1993). Stability, clearance, and disposition of intraventricularly administered oligodeoxynucleotides: implications for therapeutic application within the central nervous system. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 4665–4669.

YU, P.H. & DYCK, R.F. (1998). Impairment of methylamine clearance in uremic patients and its nephropathological implications. *Clin. Nephrol.,* **49,** 299–302.

YU, P.H., WRIGHT, S., FAN, E.H., LUN, Z.R. & GUBISNE-HARBERLE, D. (2003). Physiological and pathological implications of semicarbazide-sensitive amine oxidase. *Biochim. Biophys. Acta,* **1647,** 193–199.

ZEISEL, S.H. & DA COSTA, K.A. (1986). Increase in human exposure to methylamine precursors of *N*-nitrosamines after eating fish. *Cancer Res.,* **46,** 6136–6138.

ZIELINSKA, M., HILGIER, W., LAW, R.O., GORYNSKI, P. & ALBRECHT, J. (2002). Effects of ammonia and hepatic failure on the net efflux of endogenous glutamate, aspartate and taurine from rat cerebrocortical slices: modulation by elevated K^+ concentrations. *Neurochem. Int.,* **41,** 87–93.

(Received November 21, 2003
Revised January 29, 2004
Accepted February 12, 2004)