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Elevated circulating levels of anandamide after administration of the transport inhibitor, AM404

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Abstract

The biological actions of the endogenous cannabinoid anandamide are terminated by carrier-mediated transport into neurons and astrocytes, followed by enzymatic hydrolysis. Anandamide transport is inhibited by the compound *N*-(4-hydroxyphenyl)arachidonylamide (AM404). AM404 potentiates several responses elicited by administration of exogenous anandamide, suggesting that it may also protect endogenous anandamide from inactivation. To test this hypothesis, we studied the effects of AM404 on the plasma levels of anandamide using high-performance liquid chromatography/mass spectrometry (HPLC/MS). Systemic administration of AM404 (10 mg kg^{-1} intraperitoneal, i.p.) caused a gradual increase of anandamide in rat plasma, which was significantly different from untreated controls at 60 and 120 min after drug injection. In plasma, both AM404 and anandamide were associated with a plasma protein, which we identified as albumin by non-denaturing polyacrylamide gel electrophoresis. AM404 (10 mg kg^{-1}, i.p.) caused a time-dependent decrease of motor activity, which was reversed by the cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · hydrochloride (SR141716A, 0.5 mg kg^{-1}, i.p). These results are consistent with the hypothesis that AM404 inhibits anandamide inactivation in vivo.

Keywords: Cannabinoid; Anandamide; Fatty acid ethanolamides; High-performance liquid chromatography; Mass spectrometry; Anandamide transport

1. Introduction

Anandamide is an endogenous derivative of arachidonic acid that binds with high affinity to cannabinoid receptors and mimics virtually all pharmacological actions of plant-derived or synthetic cannabinoid drugs (Devane et al., 1992). Anandamide may be produced physiologically through enzymatic cleavage of the phospholipid precursor, *N*-arachidonyl-phosphatidylethanolamine (Di Marzo et al., 1994; Cadas et al., 1996; Sugiura et al., 1996; Cadas et al., 1997), a reaction that may be triggered by the stimulation of neurotransmitter receptors (Di Marzo et al., 1994; Giuffrida et al., 1999). After release, anandamide is disposed of through a rapid inactivation process consisting of uptake into cells (Beltramo et al., 1997; Hillard et al., 1997) followed by catalytic hydrolysis (Désarnaud et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). Anandamide uptake is a Na^+-independent process that fulfills four key criteria that define carried-mediated transport: high affinity, temperature dependence, substrate selectivity and substrate saturation (Beltramo et al., 1997; Hillard et al., 1997). Moreover, anandamide transport may be pharmacologically inhibited by several compounds, including bromcresol green and *N*-(4-hydroxyphenyl)arachidonylamide (AM404) (Beltramo et al., 1997; Hillard et al., 1997). The ability of AM404 to block anandamide transport has been demonstrated in vitro by using rat brain neurons in primary culture (Beltramo et al., 1997), human astrocytoma cells (Piomelli et al., 1999), and rat brain slices (Beltramo et al., 2000). Furthermore, in vivo experiments have shown that AM404 increases and prolongs several pharmacological responses elicited by exogenous anandamide (e.g., analgesia Beltramo et al., 1997 and hypotension Calignano et al.,

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1997a). Despite its structural similarities with anandamide, AM404 does not appear to produce a complete spectrum of cannabimimetic responses when administered alone. For example, AM404 does not elicit significant catalepsy or analgesia in rats (Beltramo et al., 2000) and hypotension in guinea pigs (Calignano et al., 1997a), three pharmacological hallmarks of direct-acting cannabinoid agonists (Pertwee, 1997). This lack of overt cannabimimetic activity is consistent with the low affinity of AM404 for cannabinoid CB_1 receptors (Beltramo et al., 1997; Beltramo et al., 2000). Although AM404 does not appear to act as a direct cannabinoid agonist, some of its pharmacological actions may resemble those of anandamide. For example, AM404 exerts significant inhibitory effects on locomotor activity, and alleviates the hyperactivity/stereotypy evoked by the dopamine receptor agonists, quinpirole and apomorphine (González et al., 1999; Beltramo et al., 2000). These effects, which are reversed by the cannabinoid CB_1 receptor antagonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide · hydrochloride (SR141716A), have been suggested to result from the ability of AM404 to block anandamide inactivation and cause it to accumulate in tissues (Beltramo et al., 2000). As a test of this hypothesis, in the present study we have determined the effects of systemic administration of AM404 on the circulating levels of anandamide in rats. Our results indicate that AM404 causes a time-dependent increase of peripheral anandamide, which is accompanied by a reduction in locomotor activity.

2. Materials and methods

2.1. Chemicals

Fatty acyl chlorides (5,8,11,14-eicosatetraenoylchloride, hexadecanoylchloride and 9-*cis*-octadecenoylchloride) were from Nu-Check Prep (Elysian, MN); $[^2H_4]$-labeled ethanolamine (isotopic atom enrichment = 98%) was from Cambridge Isotope Laboratories (Andover, MA); AM404 was from Tocris Cookson (Ballwin, MO); SR141716A was provided by RBI (Natick, MA) as part of the Chemical Synthesis Program of the National Institute of Mental Health (NIMH) (N01MH30003). All solvents were from Burdick and Jackson (Muskegon, MI) and all other chemicals from Sigma (St. Louis, MO).

2.2. Synthesis of unlabeled and $[^2H_4]$-labeled standards

Standard unlabeled and $[^2H_4]$-labeled acylethanolamides were synthesized by the reaction of the corresponding fatty acyl chlorides with unlabeled or $[^2H_4]$-labeled ethanolamine, respectively (Giuffrida and Piomelli, 1998). This reaction results in the quantitative formation of acylethanolamides (Devane et al., 1992; Giuffrida and Piomelli, 1998), which were concentrated to dryness under a stream of N_2 and reconstituted in chloroform at a concentration of 20 mM. Acylethanolamide solutions were stored at −20°C until used. Identity and chemical purity (> 98%) of the synthesized acylethanolamides were determined by thin-layer chromatography (TLC) using chloroform/methanol/ammonia (85:15:1, vol/vol) as solvent system, and subsequently by high-performance liquid chromatography/mass spectrometry (HPLC/MS).

2.3. Collection and preparation of rat plasma

Blood (2 ml) was collected from the heart of male Sprague–Dawley rats anesthetized with methoxyflurane (Schering-Plough, Union, NJ) using a syringe filled with 1 ml of Krebs–Tris buffer (in mM: NaCl 136, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 10, Trizma base 20; pH 7.4) containing 4.5 mM EDTA. Blood samples were drawn at 0, 30, 60, and 120 min after administration of a single dose of AM404 (10 mg kg^{-1} intraperitoneal, i.p.), and centrifuged in Accuspin tubes (Sigma) at $800 \times g$, for 10 min at 22°C. After centrifugation, the plasma layers were recovered and spiked with 500 pmol of $[^2H_4]$-labeled acylethanolamides. Plasma proteins were precipitated by adding cold acetone (−20°C, 1 vol) and removed by centrifugation at $1000 \times g$ for 10 min. The supernatants were flushed with a stream of N_2 to evaporate acetone and subjected to lipid extraction with methanol/chloroform (1:2, vol/vol). The recovered chloroform phases were evaporated to dryness under N_2, reconstituted in a mixture of chloroform/methanol (1:3, 80 μl), and injected into the HPLC/MS for analysis and quantification (see below). The estimated recoveries of anandamide, palmitylethanolamide and oleylethanolamide were 98.7 ± 0.2%, 78.1 ± 0.8%, and 99.7 ± 0.3%, respectively. In parallel experiments, we also measured AM404 levels in plasma by HPLC/MS. However, because of the lack of an appropriate internal standard, the recovery of AM404 was consistently lower than that of the acylethanolamides (5.2 ± 0.5%, $n = 4$). HP 1100 Series HPLC/MS system equipped with a Hewlett-Packard octadecylsilica (ODS) Hypersil column (100 × 4.6 mm i.d., 5 μm) was used. Reversed-phase separations were carried out by using linear increases of methanol (B) in water (A) (25% A, 75% B for 2 min; 15% A, 85% B for 3 min; 5% A, 95% B for 20 min; 100% B for 5 min) at a flow rate of 0.5 ml/min as described (Giuffrida et al., 2000b). Under these conditions, analytes eluted from the column with the following retention times: anandamide, 15.4 min; palmitylethanolamide, 17.3 min; oleylethanolamide, 18.4 min; AM 404, 15.9 min. MS analyses were performed in the positive ionization mode with an electrospray ion source. Capillary voltage was set at 3.0 kV, and fragmentor voltage was 80 V. Nitrogen was used as drying gas at a flow rate of 12 l/min. The drying gas temperature was set at 350°C and the nebulizer pressure at 50 psi. For quantitative analyses, diagnostic fragments corresponding to the protonated

molecules ($[M + H]^+$) and to the sodium adducts of the molecular ions ($[M + Na]^+$) were followed in the selected ion monitoring (SIM) mode. System control and data evaluation were conducted using an on-line system software (HP Chemstation).

2.4. Polyacrylamide gel electrophoresis (PAGE) / radiobinding assay

[^{3}H]anandamide (10 nM, 60 Ci/mmol) or [^{3}H]AM404 (10 nM, 200 Ci/mmol) (ARC, St. Louis, MO) were added to 10 mM potassium phosphate buffer (pH 7.4) containing either rat plasma (0.1 ml) or 70 μM bovine serum albumin (fraction V, Sigma) and incubated for 30 min at 37°C. The incubations were stopped by adding 0.1 ml of a suspension of ice-cold Dextran VI (1:1 vol/vol, Sigma). Dextran was precipitated by centrifugation and 0.1 ml of supernatant were subjected to vertical slab gel electrophoresis (PAGE, 7.5% acrylamide) under non-denaturing conditions (Siegenthaler, 1990). The gel was cut into 2-mm bands, which were incubated for 3 h in 0.5-ml Solvable (Packard, Meriden, CT) at 50°C. Radioactivity was measured by liquid scintillation counting.

2.5. Behavioral tests

We studied the effects of AM404 (10 mg kg^{-1}, i.p.), vehicle (0.9% saline containing 10% dimethyl sulfoxide, i.p.), and AM404 plus SR141716A (0.5 mg kg^{-1}, i.p.) on immobility and horizontal locomotor activity in two groups of male rats, differing in breeding, age and weight. The first group consisted of Wistar rats, 90–106 days/450–500 g (Charles River Laboratories, Wilmington, MA); the second group consisted of Sprague–Dawley rats, 56–70 days/250–300 g (Taconic, Germantown, NY). Motor activity was studied in a Digiscan photocell activity cage (42 × 42 × 30 cm) (Omnitech Electronics, Columbus, OH) equipped with 16 photocells (eight placed along the *X*, and eight along the *Y* axis of the cage) and interfaced to a microcomputer (Digiscan Analyzer) that recorded activity automatically. On the experimental day, the animals were placed in the cage, and immobility (difference between sample time and time spent moving) and horizontal activity (distance traveled by the animal in a given sample period) were recorded at 5-min intervals. This procedure was performed at 5, 30, 60 and 120 min after the injection of either vehicle or drug. SR141716A was injected 30 min before the beginning of the experiment. All behavioral tests were conducted in a sound-isolated room, illuminated with an indirect halogen light (125 lx).

2.6. Data analysis

Results are expressed as means ± S.E.M. Statistical significance was evaluated using analysis of variance (ANOVA) followed by the Dunnett's test.

3. Results

3.1. Mass spectral properties of AM404

AM404 is a structural analog of anandamide, characterized by a highly hydrophobic carbon chain and a polar carboxamido group carrying a hydroxyphenyl moiety (Fig. 1A). The mass spectral properties of AM404 were investigated by using reversed-phase HPLC/MS in a mobile phase of methanol/water. Mass spectra were acquired in the positive-ionization mode, because the total ion current (TIC) yielded by this ionization procedure was significantly higher than that obtained by negative ionization (data not shown). Fig. 1B shows a representative positive-ion electrospray spectrum of AM404. The spectrum consists of two main fragments: the protonated molecule ($[M + H]^+$, *m/z* 396.3), and the Na^+ adduct of the molecular ion ($[M + Na]^+$, *m/z* 418.2). Both ions are accompanied by X + 1 ^{13}C isotope peaks of expected abundance (McLafferty and Turecek, 1993). Additional informative

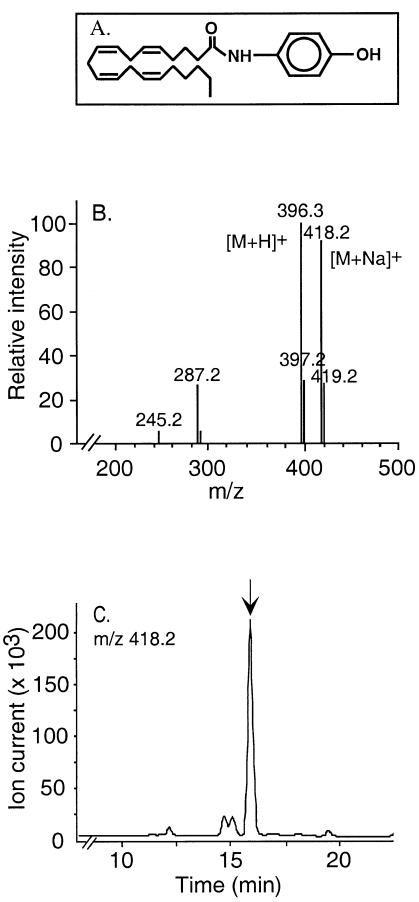

Fig. 1. Identification of AM404 in rat plasma. (A) Chemical structure of AM404. (B) Electrospray mass spectrum of AM404 (positive ions). (C) Representative selected ion recording of a rat plasma sample, showing a component of mass-to-charge ratio 418.2, which elutes from the HPLC column at the same retention time of standard AM404 (indicated by the arrow).

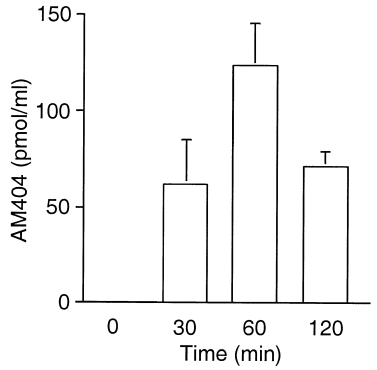

Fig. 2. Time course of AM404 accumulation in rat plasma after i.p. administration of a single dose of the drug (10 mg kg^{-1}). Values are means ± S.E.M. of six independent experiments.

fragments are m/z 287.2 ($[M-108]^+$, loss of $NH–C_6H_4–OH$) and m/z 245.2 ($[M-150]^+$, possibly corresponding to the loss of $CH_2–CO–NH–C_6H_4–OH$).

3.2. Quantification of AM404 in plasma

AM404 analysis was carried out by monitoring the $[M + Na]^+$ fragment (m/z 418.2) in the SIM mode (Fig. 1C). This ion, although slightly less abundant than the $[M + H]^+$ fragment, was selected because of its greater resolution from contaminating components present in plasma samples. For quantification purposes, a calibration curve was constructed by injecting into the HPLC/MS increasing amounts of AM404. The areas obtained from the integration of SIM peaks were plotted against the injected amounts. Under these conditions the MS responses were linear ($r^2 = 0.93$; $n = 3$) over the range 0–250 pmol (data not shown).

Blood samples were collected by cardiac puncture from rats killed 30, 60, and 120 min following systemic (i.p.) injection of AM404 (10 mg/kg). After acetone precipitation of plasma proteins, lipids were extracted with chloroform/methanol and analyzed by HPLC/MS. All plasma

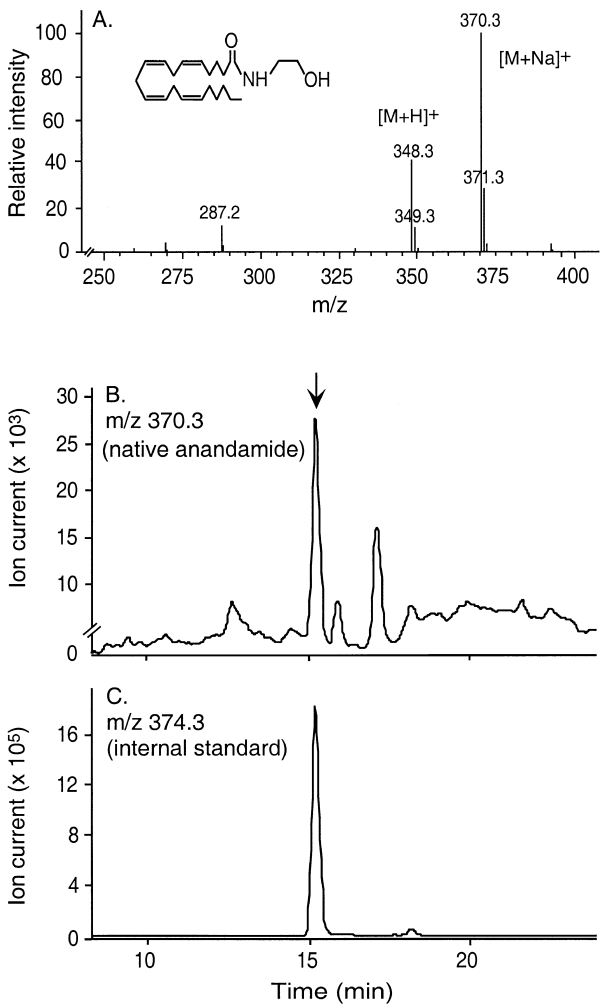

Fig. 3. Identification of anandamide in rat plasma. (A) Chemical structure and electrospray mass spectrum of anandamide (positive ions). (B and C) Representative selected ion tracings of a rat blood plasma sample showing endogenous (B) and synthetic ($[^2H_4]$-anandamide (C). The arrow indicates the retention time of standard anandamide.

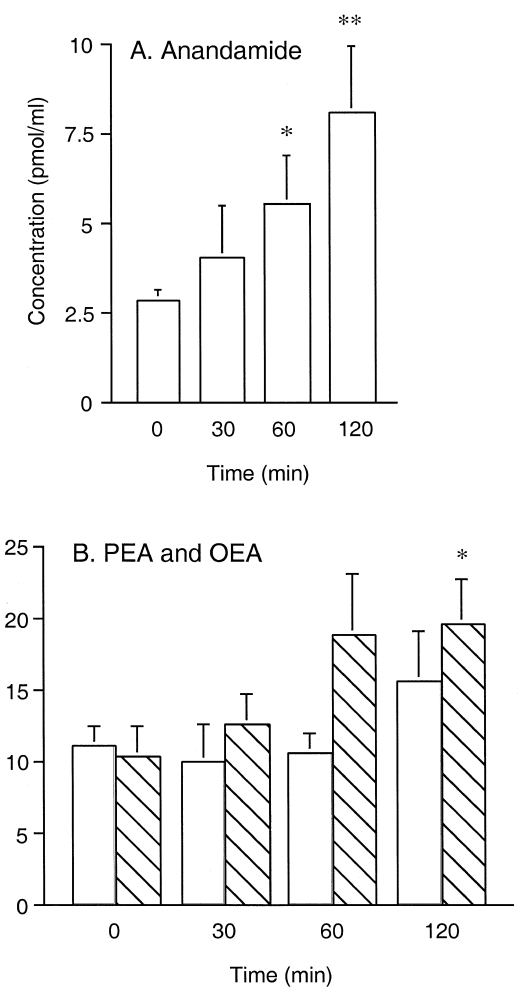

Fig. 4. Time-course of the effects of systemic administration of AM404 (10 mg kg^{-1}) on the plasma levels of (A) anandamide and (B) palmitylethanolamide (open bars) and oleylethanolamide (hatched bars). Values are means ± S.E.M. of seven independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. controls.

samples displayed a peak eluting from the column at the retention time of standard AM404 (15.9 min) (Fig. 1C). The plasma concentrations of AM404, illustrated in Fig. 2, increased gradually reaching a maximal value (123 ± 22 pmol/ml) at 60 min after injection, and declining thereafter. As expected of a hydrophobic molecule, the decline of AM404 in plasma was accompanied by a substantial accumulation of the compound in brain tissue (data not shown).

3.3. Quantification of anandamide in plasma

To determine whether the systemic administration of AM404 results in accumulation of endogenously produced anandamide, we measured the plasma levels of anandamide after a bolus injection of AM404 (10 mg kg^{-1}, i.p.). In the same samples, we measured the levels of two additional fatty acid ethanolamides, palmitylethanolamide and oleylethanolamide, which do not activate cannabinoid receptors. Quantification was carried out by monitoring the $[M + Na]^+$ ions with the following m/z values: for anandamide and $[^2H_4]$-anandamide, m/z 370.3 (Fig. 3A) and 374.3, respectively; for palmitylethanolamide and $[^2H_4]$-palmitylethanolamide, m/z 322.3 and 326.3; for oleylethanolamide and $[^2H_4]$-oleylethanolamide, m/z 348.3 and 352.3.

All plasma samples contained lipid components that eluted from the HPLC at the retention times expected for anandamide, palmitylethanolamide and oleylethanolamide (Fig. 3B and data not shown). In samples collected immediately (1 min) after AM404 administration, we measured 2.8 ± 0.3 pmol/ml of anandamide, 11 ± 1.4 pmol/ml of palmitylethanolamide and 10.3 ± 2.1 pmol/ml of oleylethanolamide ($n = 12$) (Fig. 4A,B). These levels are identical to those observed in rats that received no drug injection (Giuffrida et al., 2000b and data not shown). Anandamide levels in plasma increased gradually after AM404 administration, and were significantly different from controls 60 min (5.5 ± 1.4 pmol/ml, $n = 7$) and 120 min (8.1 ± 1.8 pmol/ml, $n = 7$) after injection of the drug (Fig. 4A). By contrast, the plasma levels of palmitylethanolamide were not affected by AM404, while those of oleylethanolamide showed a trend towards accumulation that was statistically different from controls only 120 min after injection (19.6 ± 3.1 pmol/ml) (Fig. 4B).

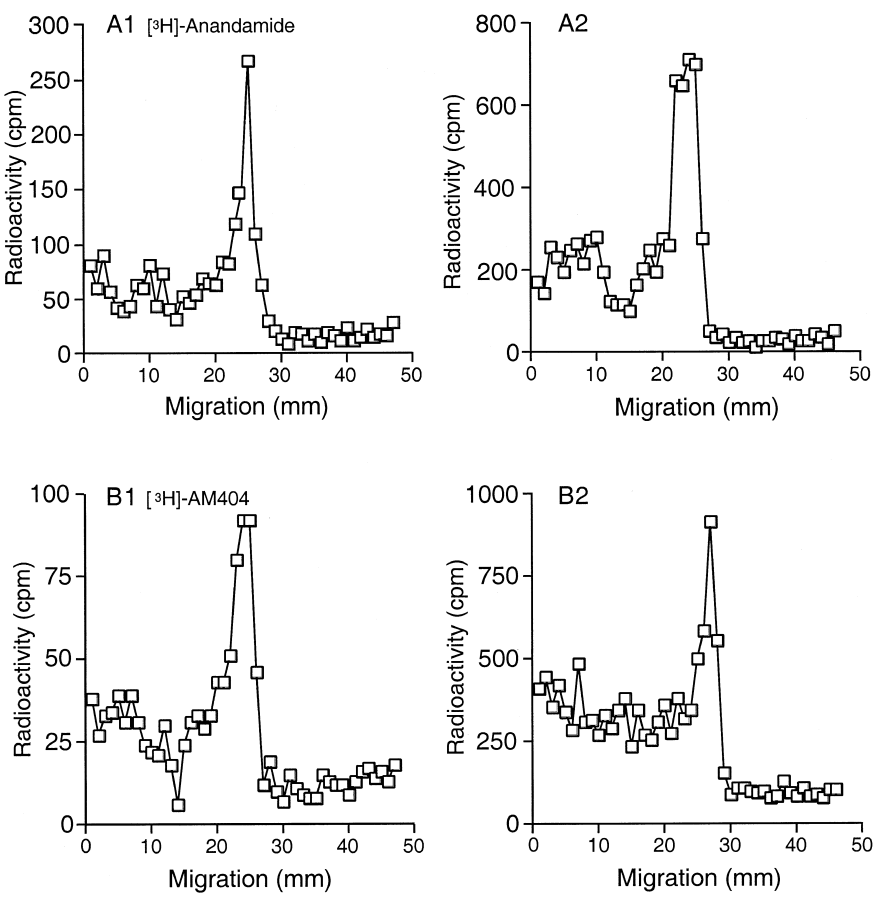

Fig. 5. PAGE analysis under non-denaturing conditions of rat plasma and bovine serum albumin incubated with $[^3H]$-anandamide (A1 and A2, respectively) or $[^3H]$-AM404 (B1 and B2, respectively). 2-mm sections of the gel were cut, and radioactivity was measured by liquid scintillation counting.

3.4. Binding of anandamide and AM404 to plasma proteins

Anandamide and AM404 were detectable in plasma only when proteins were first denatured with cold acetone (data not shown), suggesting that these lipids may be tightly associated to plasma proteins. To test this hypothesis, [^{3}H]-labeled anandamide was incubated in plasma or in a solution containing bovine serum albumin (70 μM), and analyzed by PAGE under non-denaturing conditions. All plasma samples showed a significant radioactive peak that co-migrated on the gel with albumin (Fig. 5A1, A2). Similar results were obtained after electrophoresis of either plasma or bovine serum albumin solutions incubated with [^{3}H]AM404 (Fig. 5B1, B2).

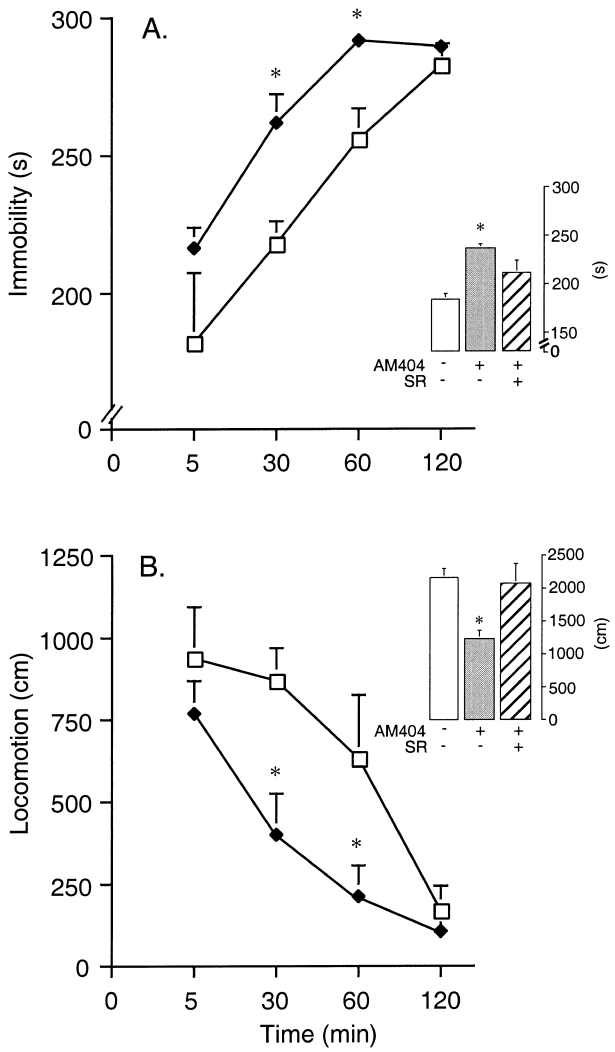

Fig. 6. Time course of the effects of i.p. administration of vehicle (squares) or AM404 (diamonds; 10 mg kg^{-1}) on time spent in immobility (A) and horizontal locomotor activity (B) in Wistar rats. The effects of vehicle (open bars), AM404 (filled bars; 10 mg kg^{-1}, i.p.) or cannabinoid CB_1 receptor antagonist SR141716A (SR, 0.5 mg kg^{-1}, i.p.) plus AM404 (hatched bars) on immobility (A, inset) and locomotor activity (B, inset) at 60 min after drug applications were also monitored in Sprague–Dawley rats. SR141716A was administered 60 min before AM404. Values are means ± S.E.M. of six independent experiments. * $P < 0.05$ compared to vehicle.

3.5. Effects of AM404 on motor behavior

As previously reported (González et al., 1999; Beltramo et al., 2000), systemic administration of AM404 (10 mg/kg, i.p.) caused a time-dependent decrease in motor activity, which was measured either as increase of time spent in total immobility (Fig. 6A), or as reduction of horizontal locomotion (Fig. 6B). This effect was statistically significant 30 and 60 min after drug administration. Comparable inhibitory effects on motor behaviors were observed in a second group of animals differing from the previous in breeding, age and weight (Fig. 6, inset). In the latter group, we also tested the effects of the selective cannabinoid CB_1 receptor antagonist SR141716A. Administration of SR141716A (0.5 mg/kg, i.p.) 60 min before AM404 did not cause any change in basal motor activity (data not shown), but reversed the effects of AM404 on immobility (Fig. 6A, inset) and locomotor activity (Fig. 6B, inset).

4. Discussion

Drugs that interfere with anandamide transport have both theoretical and practical interest. Theoretically, they may help uncover the functions of the endocannabinoid system, which remain largely uncharacterized. Practically, they may find applications in a range of therapeutic areas, such as pain, multiple sclerosis and motor disorders (Piomelli et al., 2000; Baker et al., 2000; Giuffrida et al., 2000a).

AM404—the first synthetic inhibitor of anandamide uptake (Beltramo et al., 1997)—has been shown to potentiate many effects elicited by anandamide in vitro (Beltramo et al., 1997) and in vivo (Beltramo et al., 1997; Calignano et al., 1997a). Because of the inability of AM404 to activate cannabinoid receptors (Beltramo et al., 1997, 2000), the effects of this drug were suggested to result from the elevation of endogenous anandamide levels. However, this hypothesis was solely based on pharmacological evidence and was not supported by direct measurements of endogenous anandamide levels after AM404 administration. In the present study, we show that systemic administration of AM404 causes an increase in the concentration of circulating anandamide. This was accompanied by an elevation of AM404 levels in plasma, which reached a peak value of 123 ± 22 pmol/ml and remained as high as 70.5 pmol/ml for at least 2 h after drug administration. These values are likely to reflect the amount of AM404 bound to plasma proteins. Indeed, we found that both AM404 and anandamide bind to a protein in plasma, which we identified as albumin by non-denaturing PAGE.

The concentrations of AM404 at its sites of action, i.e., at the membranes of cells expressing the putative anandamide transporter, remain at present undetermined.

To further investigate the biochemical effects of AM404 in vivo, we also monitored the plasma levels of palmitylethanolamide and oleylethanolamide, two fatty acylethanolamides that are produced through the same biosynthetic mechanism of anandamide (Di Marzo et al., 1994; Cadas et al., 1997), but do not serve as substrates for the anandamide transporter Piomelli et al. (1999). AM404 administration did not significantly affect the levels of palmitylethanolamide, but caused a slow increase of circulating oleylethanolamide statistically significant 120 min after AM404 injection. Since oleylethanolamide is not transported by anandamide carrier, a possible interpretation of this result is that AM404 may inhibit an as-yet uncharacterized transporter of oleylethanolamide. Alternatively, oleylethanolamide elevation may result from the interference of AM404 with anandamide amidohydrolase, of which oleylethanolamide represents a substrate. However, administration of the potent anandamide amidohydrolase blocker, palmitylsulfonyl fluoride (AM374) (Gifford et al., 1999) had no effect on circulating anandamide levels, although it significantly increased the levels of oleylethanolamide 30 min after drug application (A. Giuffrida, F. Nava, A. Makriyannis and D. Piomelli, in preparation). Taken together, these results suggest that blockade of anandamide amidohydrolase activity is unlikely to participate in the elevation of anandamide in plasma, but may cause the accumulation of other fatty acid acylethanolamides.

In parallel with its ability to increase anandamide levels in plasma, AM404 also elicited a time-dependent inhibition of motor activity. The hypokinetic effect of AM404 is reminiscent of that observed after anandamide administration (Fride and Mechoulam, 1993; Smith et al., 1994; Romero et al., 1995) and was reversed by the cannabinoid CB_1 receptor antagonist SR141716A. This drug prevents several responses of anandamide in rats (Calignano et al., 1998), mice (Beltramo et al., 1997; Calignano et al., 1997b) and guinea pigs (Calignano et al., 1997a). However, failure of SR141716A to reverse anandamide actions was also reported (Adams et al., 1998). The reason for these discrepancies are unresolved, and may be due to differences in species, dosage of the drug and experimental design.

Since the motor inhibition produced by AM404 was achieved at a dose that also caused accumulation of anandamide in peripheral blood, and given the inability of AM404 to activate cannabinoid receptors (Beltramo et al., 1997, 2000), our results are consistent with the hypothesis that AM404 produced its behavioral effects by protecting endogenous anandamide from transport-mediated inactivation. Alternatively, the effects of AM404 may be ascribed to its ability to activate vanilloid VR1-type receptors (Zygmunt et al., 2000; Smart and Jerman, 2000; Jerman et al., 2000). This possibility is unlikely for two reasons: (1) the effects of AM404 were prevented by the highly selective cannabinoid CB_1 receptor antagonist, SR141716A, which does not interfere with vanilloid VR1 receptor function (Calignano et al., 2000); (2) injection of vanilloid agonists into the brain does not cause hypolocomotion (Mezey et al., 2000), the primary effect of AM404 observed in the present and other studies (Beltramo et al., 2000; González et al., 1999).

Although the concentrations reached by anandamide in plasma (approximately 10 nM) are insufficient to activate cannabinoid receptors (K_d = 50 nM and 1.6 μM for CB_1 and CB_2 receptor, respectively) (Pertwee, 1997), it is reasonable to hypothesize that AM404 may cause anandamide to accumulate in brain tissue to an extent that is sufficient to cause biological effects. This would explain why the motor inhibition elicited by AM404 takes place before significant accumulation of anandamide in plasma is observed. However, the sources of plasma anandamide following AM404 administration are still unknown. Indeed, anandamide production has been demonstrated not only within the central nervous system, but also in peripheral cells, such as macrophages and platelets (Schmid et al., 1997; Wagner et al., 1997). Microdialysis studies aimed at measuring anandamide outflow in brain areas involved in motor control after AM404 administration should shed light in this important question.

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