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MICROSOMAL CYTOCHROME P450-MEDIATED LIVER AND BRAIN ANANDAMIDE METABOLISM

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Abstract—Anandamide (AN) is an arachidonic acid congener, found in the brain, that binds to the cannabinoid receptor and elicits cannabinoid-like pharmacological activity. Cytochromes P450 (P450s) are known to oxidize arachidonic acid to a wide variety of metabolites, yielding many physiologically potent compounds. To determine if AN could be similarly oxidized by P450s, its metabolism by mouse liver and brain microsomes was examined. Mouse hepatic microsomal incubation of AN with NADPH resulted in the generation of at least 20 metabolites, determined after HPLC separation by increased UV-absorbance at 205 nm. Pretreatment of mice with various P450 inducers resulted in increased hepatic microsomal formation of several AN metabolites, with dexamethasone being the most effective inducer. Phenobarbital pretreatment resulted in a metabolic profile similar to that observed after dexamethasone pretreatment, whereas 3-methylcholanthrene pretreatment selectively increased the formation of several other metabolites. Clofibrate pretreatment had no effect on hepatic AN metabolism. Polyclonal antibodies prepared against mouse hepatic P450 3A inhibited the formation of several AN metabolites by hepatic microsomes from untreated mice as well as the formation of those metabolites found to be increased after dexamethasone pretreatment. AN metabolism by brain microsomes resulted in the formation of two NADPH- and protein-dependent metabolites. Hepatic P450 3A antibody partially inhibited the formation of only one of these metabolites. Thus, P450 3A is a major contributor to AN metabolism in the liver but not in the brain. The physiological consequences of P450-mediated AN metabolism remain to be determined.

Key words: anandamide; cannabinoids; cannabinoid receptor; cytochrome P450; drug metabolism; tetrahydrocannabinol

AN† has been identified recently as a brain constituent that binds selectively to a cannabinoid receptor and mimics the pharmacological actions of cannabinoids such as tetrahydrocannabinol [1–3]. AN is an amide of arachidonic acid and ethanolamine (Fig. 1) and, in common with arachidonic acid, it has the potential to be metabolized by microsomal P450s [4–6] to corresponding epoxides (EETs) and hydroxy-metabolites (HETEs and ω, ω-1, and ω-2 alcohols). Since many arachidonic acid metabolites are very potent and elicit many physiological effects [7], AN metabolism by liver and brain microsomes was studied to determine if it might be converted to potential physiologically relevant metabolites.

MATERIALS AND METHODS

Materials. AN was synthesized according to published procedures [1, 8, 9]. [^{3}H]AN (210 Ci/mmol, 5,6,8,9,11,12,14,15-^{3}H) was obtained from Dupont NEN Research Products, Boston, MA. All other chemicals were, at the least, of reagent grade.

Animal treatment. Male CF-1 mice (Charles River) were either untreated or treated intraperitoneally with dexamethasone (100 mg/kg) in corn oil, sodium phenobarbital (100 mg/kg) in saline, or clofibrate (250 mg/kg) in corn oil for 4 days. Male C57/B1 mice were either untreated or treated with 3-methylcholanthrene (20 mg/kg) in corn oil for 3 days.

Analytical procedures. Microsomes were prepared from liver and brain homogenates by differential centrifugation as described [10]. Protein concentrations were determined by the method of Lowry *et al.* [11], using bovine serum albumin as the standard.

Determination of microsomal AN hydroxylase activity. AN metabolism was determined after incubation of hepatic microsomal protein (0.3 mg) with AN (0.72 mM), phenylmethylsulfonyl fluoride (3 mM) and NADPH (1 mM) in 0.1 M phosphate buffer, pH 7.4, containing diethylenetriaminepentaacetic acid (1 mM) in a total volume of 1 mL at 37° for 15 min. Reactions were terminated by the addition of ethyl acetate (2 mL) containing 0.01% butylated hydroxytoluene. After extraction of incubations with ethyl acetate, metabolites were separated by reverse-phase HPLC (Rainin Dynamax C18 column, 4.6 × 250 mm, Woburn, MA) with 40% acetonitrile for 10 min, followed by a linear gradient of 40–70% acetonitrile over 10 min, and

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† Abbreviations: AN, anandamide; P450s, cytochromes P450; HETE, hydroxyeicosatetraenoic acid; and IgG, immunoglobulin G.

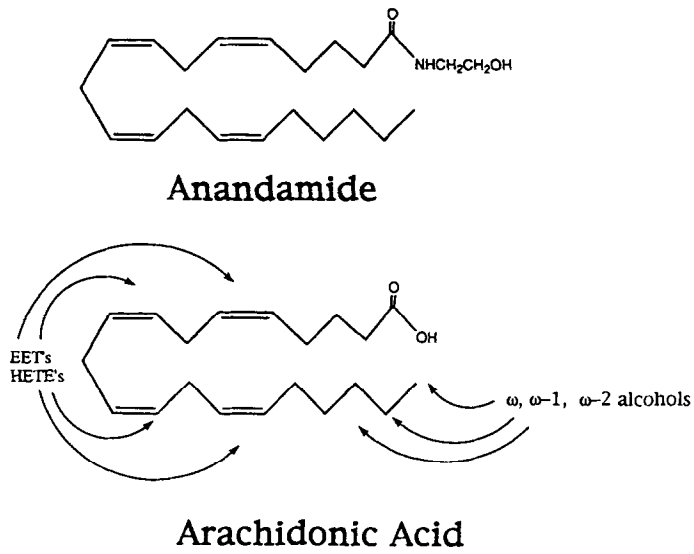

Fig. 1. Chemical structures of AN and arachidonic acid. Known sites of arachidonic acid metabolism are indicated by arrows.

then a 100% acetonitrile wash. Metabolites were quantitated by their absorbance at 205 nm, assuming extinction coefficients comparable to that of AN.

Brain AN hydroxylase activity was determined similarly except that microsomal protein was increased to 2 mg and incubation times were increased to 45 min.

For the radiometric determination of AN metabolism, hepatic microsomal protein was incubated as described above except that 1 μCi of [^{3}H] AN was added to the incubation and metabolites were separated by reverse-phase HPLC with 45% acetonitrile for 10 min, followed by a linear gradient of 45–75% acetonitrile over 10 min, before the column was washed with 100% acetonitrile.

Immunoinhibition of AN hydroxylase activity. Immunoinhibition studies were performed after preincubation of microsomes at 4° for 15 min with antibody [12] raised against mouse constitutive hepatic microsomal P450 3A (0.5 to 10 mg antibody/mg microsomal protein).

Mass spectral analysis. Brain AN metabolites were isolated from 4-mL incubates containing 5 mg/mL of microsomal protein after incubation at 37° for 2 hr. Metabolites were purified by reverse-phase (C18) HPLC using isocratic 40% acetonitrile in water. Metabolites were characterized by LC/MS on a VG Quattro BQ mass spectrometer (Manchester, U.K.) in the positive mode by electrospray (4.0 kV tip voltage, 5 sec/scan over the mass range of 100–1000 amu) and by liquid secondary ion monitoring on a VG 70 double-focusing mass spectrometer (Manchester, U.K.) equipped with a cesium ion source.

RESULTS

AN was metabolized by mouse liver microsomes to at least 20 different metabolites, detected after HPLC separation by their increased UV-absorbance at 205 nm (Fig. 2) and/or on-line radiometric detection (Fig. 3) after incubation with [^{3}H]AN. Due to different elution conditions and resolving capabilities of UV and radiometric detection, the metabolic profiles in Figs. 2 and 3 are not directly comparable. However, it is clear that most of the peaks detected by increased UV-absorbance at 205 nm in Fig. 2 were found to be radiolabeled in Fig. 3, indicating that they were produced from radiolabeled AN. UV-absorbance detection was employed for all subsequent work because of its increased resolution of AN metabolite detection. Metabolite formation was found to be NADPH dependent, and formation rates varied from 8 to 386 pmol product/mg protein/min.

To determine the contribution of different P450s to AN metabolism, mice were pretreated with dexamethasone, phenobarbital, 3-methylcholanthrene, or clofibrate to induce P450s 3A, 2B, 1A and 4A, respectively. Dexamethasone pretreatment increased the formation of fifteen AN metabolites and decreased the formation of two others (Fig. 4A). Although these metabolites had retention times similar to those produced by hepatic microsomes from untreated mice, it cannot be concluded on that basis alone that these metabolites are identical and that the changes in metabolite formation after dexamethasone pretreatment are merely quantitative and not qualitative. However, it is clear that dexamethasone pretreatment markedly altered the quantitative profile of AN metabolites. Phenobarbital pretreatment produced effects qualitatively similar to those of dexamethasone, although they were smaller in magnitude (Fig. 4B). 3-Methylcholanthrene pretreatment increased the formation of five metabolites that were largely unaffected by

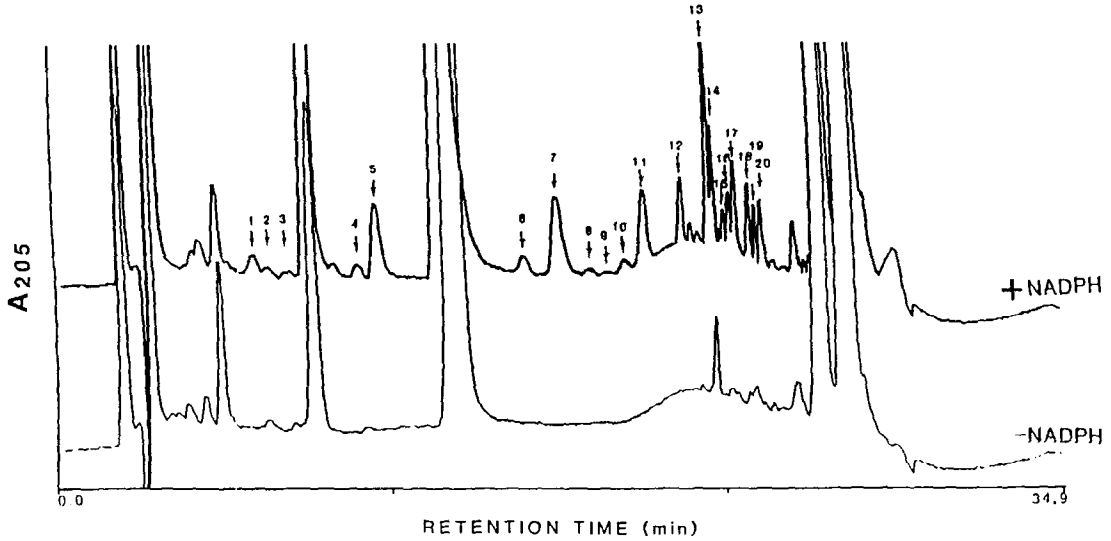

Fig. 2. Reverse-phase HPLC of AN and its hepatic microsomal metabolites. Mouse hepatic microsomes (0.3 mg protein) were incubated with AN (0.72 mM) in the presence (upper trace) or absence (lower trace) of NADPH (1 mM) at 37° for 15 min. After extraction of incubations with ethyl acetate, metabolites (1–20) were separated by reverse-phase (C18) HPLC as described in Materials and Methods. The *y*-axis represents the absorbance at 205 nm.

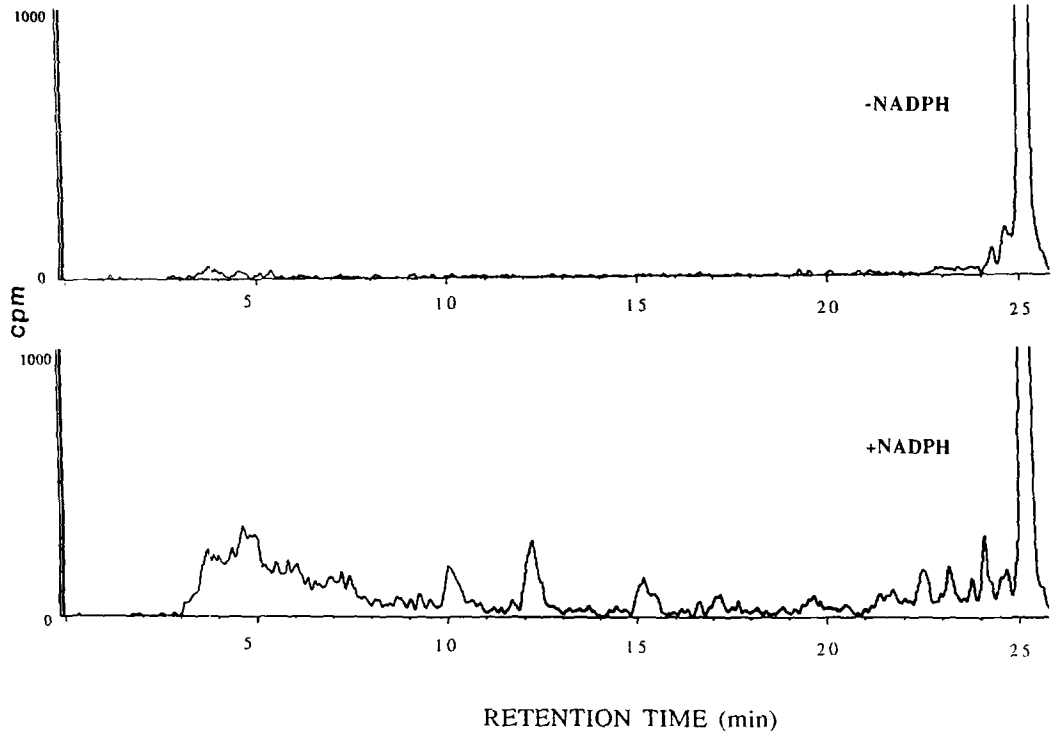

Fig. 3. Radiometric profiles of reverse-phase HPLC-separated AN and its metabolites. Mouse hepatic microsomes were incubated with [^{3}H]AN as in Fig. 2 before analysis of extracted metabolites as described in Materials and Methods by HPLC equipped with an on-line radiometric detector (Radiomatic Flow-One/Beta Detector, Tampa, FL). The upper and lower traces are from samples incubated in the absence or presence of NADPH, respectively. The *y*-axis represents the counts/min of the column eluate not corrected for quench.

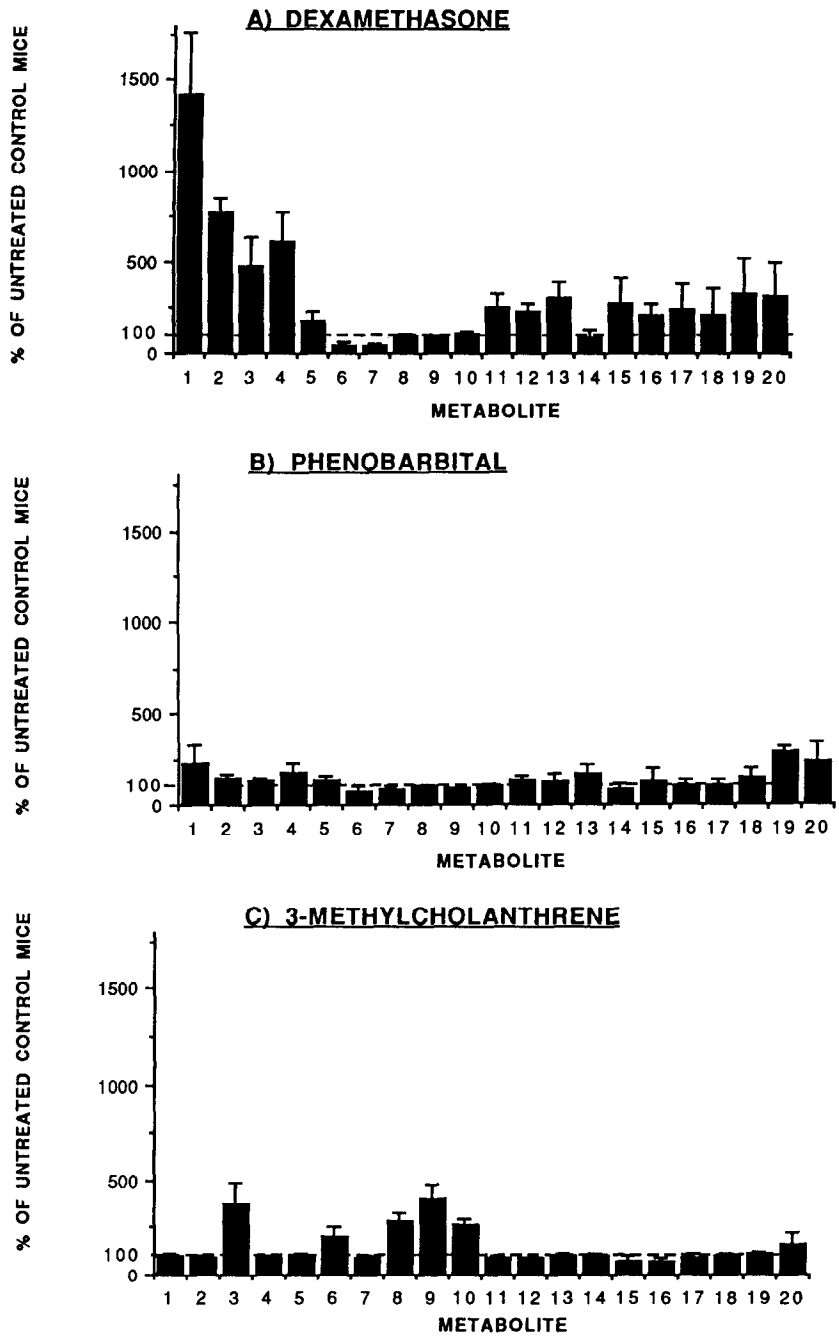

Fig. 4. Effect of P450 inducer pretreatment on hepatic microsomal AN metabolism. Mice were pretreated with dexamethasone (A), phenobarbital (B), or 3-methylcholanthrene (C) before hepatic microsomes were prepared, and AN metabolism was determined as in Fig. 2. Values are the means ± SD of at least three different microsomal preparations and are presented as the percentage of corresponding untreated controls. The untreated control microsomal metabolite values varied from 8 to 386 pmol product formed/mg protein/min.

either dexamethasone or phenobarbital pretreatment (Fig. 4C). Clofibrate pretreatment had no effect on the profile of AN metabolites (data not shown).

Antibody raised against mouse hepatic microsomal P450 3A largely inhibited (Fig. 5) those metabolites whose formation was increased by both dexamethasone and phenobarbital pretreatment (Fig. 4A and 4B). This was true irrespective of the source of hepatic microsomes: untreated (Fig. 5A) or dexamethasone-pretreated mice (Fig. 5B).

To examine AN metabolism in a tissue wherein AN is physiologically present, whole brain microsomes

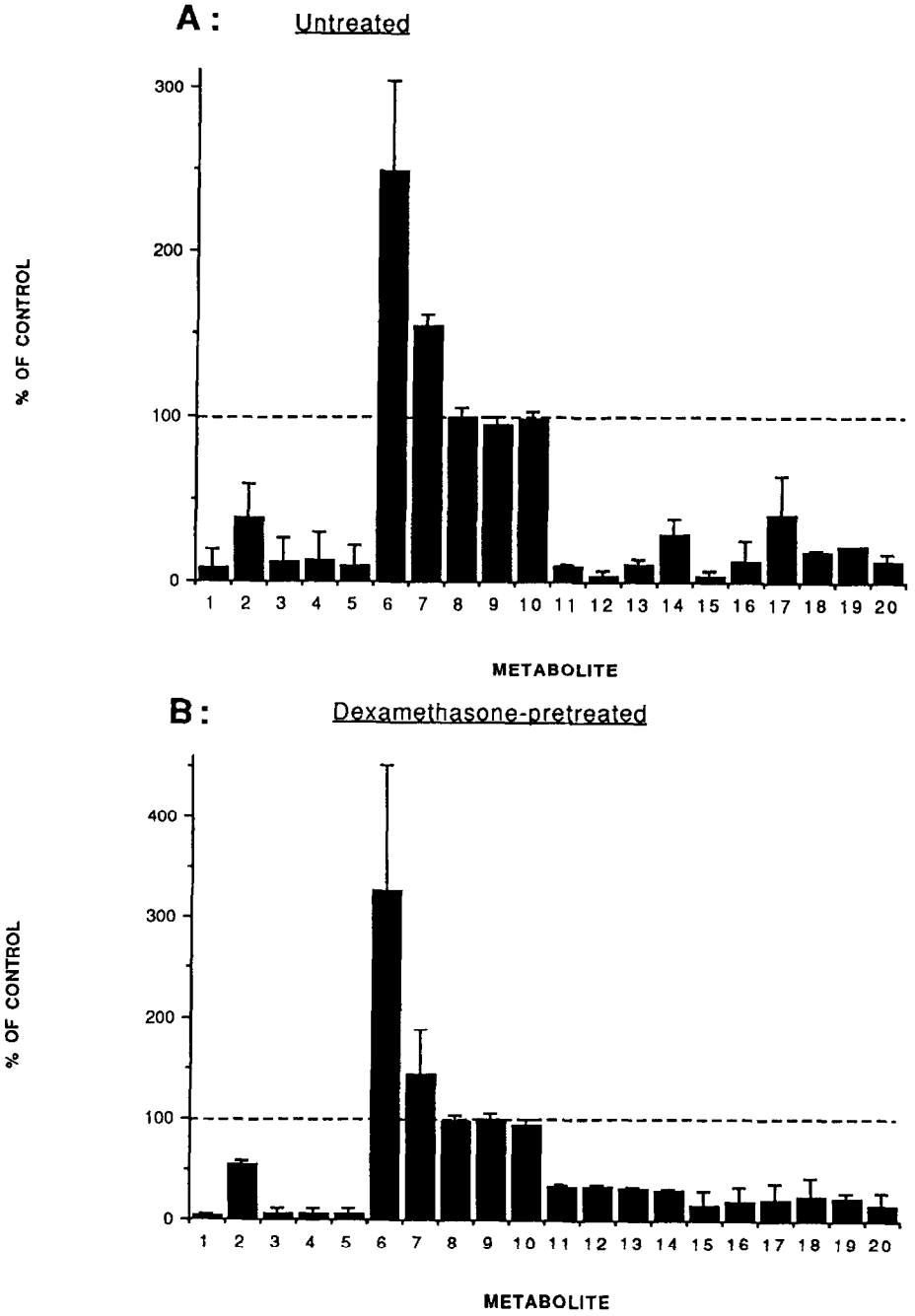

Fig. 5. Effect of P450 3A immunoinhibition on hepatic microsomal AN metabolism. AN metabolism was determined with liver microsomes from either untreated (A) or dexamethasone-pretreated (B) mice after preincubation with anti-P450 3A IgG. Values are presented as the percentage of control microsomes incubated without IgG and are the means ± SD of three different microsomal preparations. Preimmune IgG inhibited activities <10%. The control microsomal metabolite values varied from 8 to 386 pmol product formed/mg protein/min.

were prepared and incubated with AN in the presence or absence of NADPH (Fig. 6). AN was metabolized by mouse brain microsomes to two major metabolites, whose formation was both time and protein concentration dependent (Fig. 7, A and B). The rates of formation of metabolites A and B were 7 and 17 pmol/mg protein/min, respectively. The retention times of these metabolites appeared to differ from those found after hepatic AN metabolism and would elute between metabolites numbered 11 and 12 (Fig. 2). Mass spectral analysis of these brain metabolites was accomplished by

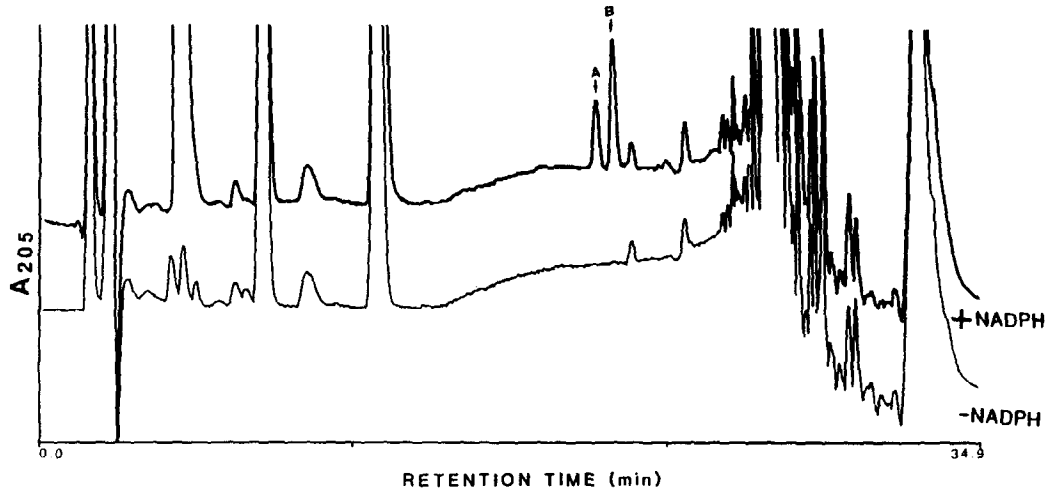

Fig. 6. Reverse-phase HPLC of AN and its brain microsomal metabolites. Mouse brain microsomes were incubated with AN before HPLC analysis of extracted metabolites (metabolites A and B) as in Fig. 2, except that 2 mg of protein was used and the incubation time was increased to 45 min. The *y*-axis represents the absorbance at 205 nm.

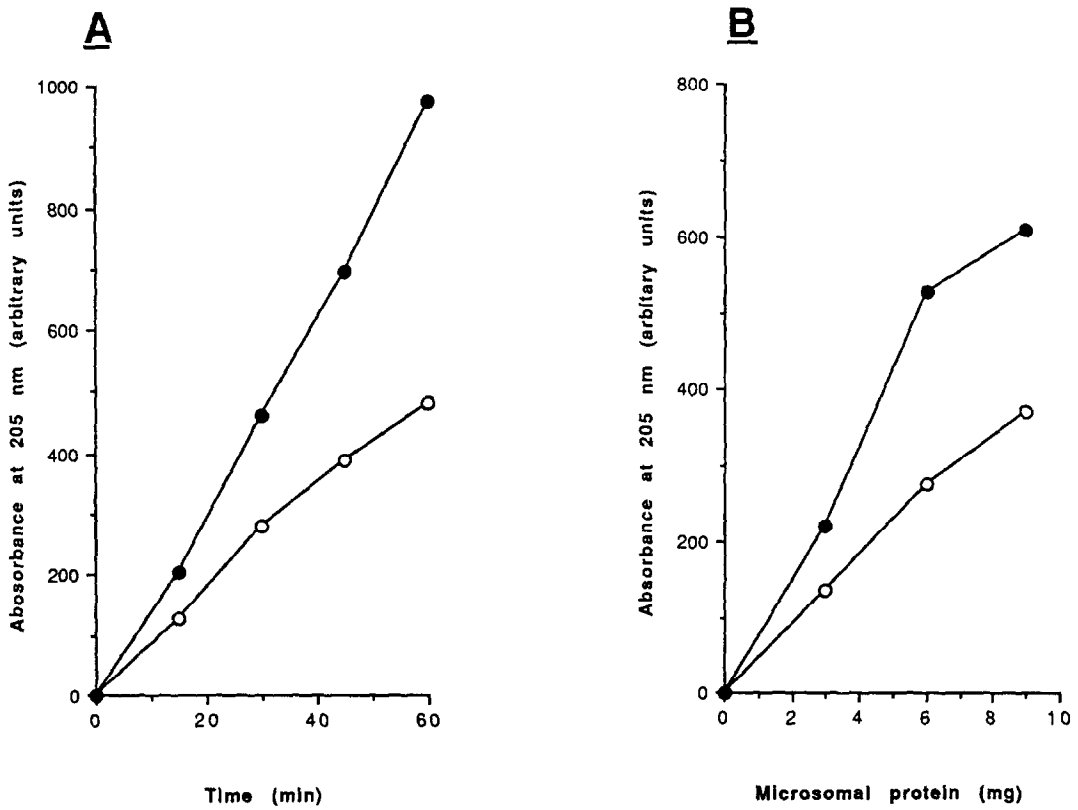

Fig. 7. Linearity of brain microsomal AN metabolism. (A) AN was incubated with brain microsomes for various periods of time before AN metabolism to metabolite A (○) and metabolite B (●) was determined as described in Materials and Methods. (B) AN was incubated with various amounts of brain microsomes for 45 min before AN metabolism was determined as above.

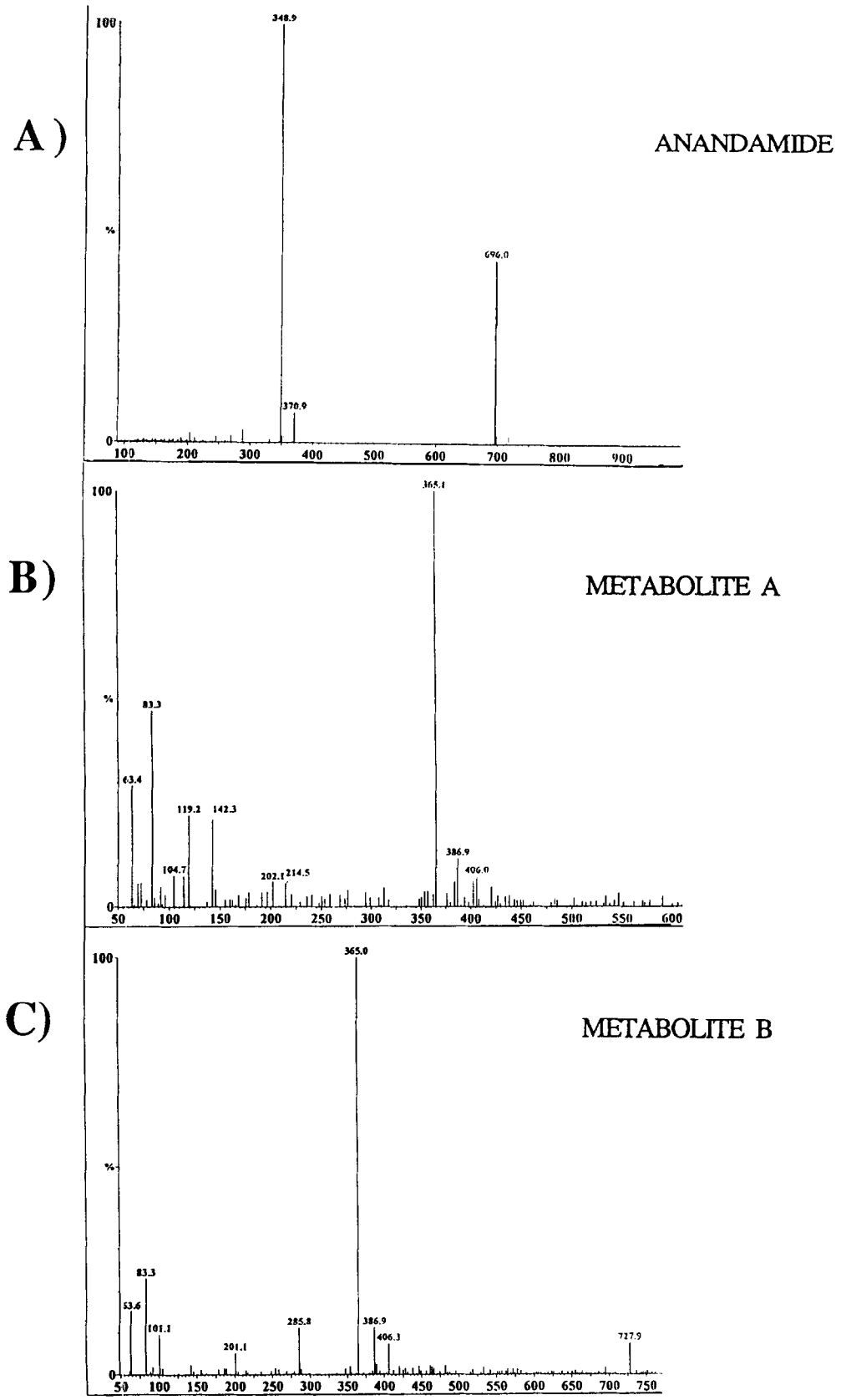

Fig. 8. Mass spectra of AN and its brain microsomal metabolites. AN metabolites from 4-mL incubates were purified by HPLC as described in Materials and Methods. Anandamide (A) and metabolites A (B) and B (C) were characterized by LC/MS electrospray on a VG Quattro BQ spectrometer in the positive ion mode. The relative ion abundance (y-axis) versus the mass/charge (x-axis) is depicted.

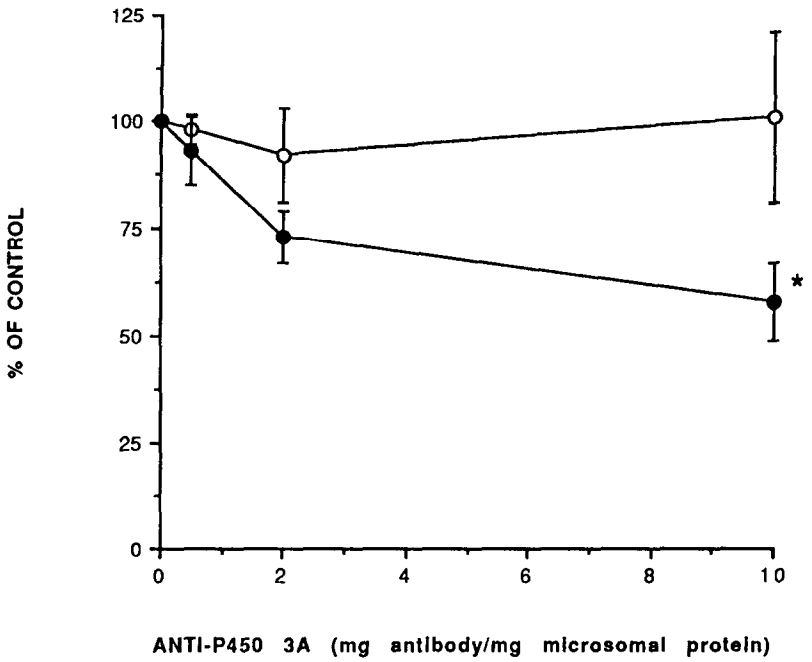

Fig. 9. Effect of P450 3A immunoinhibition on brain microsomal AN metabolism. AN metabolism to metabolite A (○) and metabolite B (●) was determined with brain microsomes after preincubation with anti-hepatic microsomal P450 3A IgG. Values are presented as the percentage of control microsomes incubated without IgG and are the means ± SEM of three different microsomal preparations. Control microsomes formed metabolites A and B at rates of 7 and 17 pmol/mg protein/min, respectively. An asterisk (*) indicates a value significantly different from control ($P < 0.05$). Preimmune IgG inhibited activities <10%.

LC/MS (Fig. 8), as well as by liquid secondary ion monitoring (data not shown). AN exhibited an MH^+ ion (m/z 349), as well as m/z 371 and 696 ions, corresponding to a sodium adduct and an AN-dimer, respectively (Fig. 8A). Mass spectral analysis of the AN metabolites (Fig. 8, B and C) revealed them to be 16 mass units higher (m/z 365) than AN, corresponding to the addition of a single atom of oxygen; small amounts of sodium adducts (m/z 387) were also detected. To determine if P450 3A was a major contributor to AN brain metabolism, brain microsomes were preincubated with antibodies raised against mouse hepatic microsomal P450 3A. P450 3A antibodies had no effect on metabolite A formation, but partially inhibited (~40%) the formation of metabolite B (Fig. 9).

DISCUSSION

AN is the first chemical to be identified in the brain that binds to the cannabinoid receptor [1] and produces many of the pharmacological effects caused by tetrahydrocannabinol [2, 3]. Two additional fatty acid ethanolamides have been identified in the brain, which also bind to the cannabinoid receptor [13], and additional endogenous ligands most likely also exist. A peripheral cannabinoid binding receptor has been identified recently in splenic macrophages, which binds cannabinoid analogs with profiles somewhat distinct from that of the brain receptor [14]. It is therefore likely that additional ligands and receptors will be identified in the future with varying affinities for each other.

AN is an arachidonic acid analog, and many stereo-specific P450-derived arachidonic acid metabolites possess different physiological activities. For instance, 12(*R*)-HETE is a potent Na^+/K^+-ATPase inhibitor and vasoconstrictor, and reduces intraocular pressure [15], whereas its dihydro-metabolite, 12(*R*)-HETrE is a potent vasodilator, and a chemotactic and angiogenic factor [16]. On the other hand, the lipoxygenase-derived 12(*S*)-HETE is devoid of much of these activities [15]. Thus, potentially, the characterization of stereo- and regio-specific AN metabolites might permit the identification of many important physiologically relevant compounds. We have demonstrated previously that AN is a substrate for hepatic microsomal P450 [9] and that cannabidiol, a cannabinoid that inactivates P450 2C and 3A in the mouse [17], decreases the formation of several AN metabolites. In the present study, we have examined the effect of P450 inducers on AN metabolism in order to more fully identify the P450 subfamilies involved. Both constitutive and steroid-inducible P450s 3A appear to contribute greatly to AN hepatic metabolism by catalyzing the formation of many different AN metabolites (Figs. 4 and 5). The large number of metabolites may be due to sequential metabolism, yielding mono- and dihydroxylated products as well as epoxides and

combinations thereof. P450s 1A catalyze the formation of fewer metabolites that largely appear to be different from those produced by P450s 3A. P450s 4A, which catalyze the formation of ω and ω-1 alcohols of fatty acids [18], do not appear to detectably metabolize AN since clofibrate pretreatment did not affect AN metabolite formation. In the latter studies, clofibrate-mediated induction of hepatic microsomal P450s 4A was confirmed by immunoblot analysis, which revealed a marked increase in liver P450 4A content.

Although liver microsomal P450s are abundant, well-characterized and easily induced by chemical means, their physiological role in endogenous AN metabolism is unclear. AN has not been identified in the liver, although AN synthetase and amidase activities have been demonstrated in the liver, brain, kidney, and lung [19, 20].

Because AN has been identified as a natural brain constituent and since the psychological effects of cannabinoids are believed to be mediated through binding to the brain cannabinoid receptor, AN metabolism in the brain might be of great physiological relevance. We have demonstrated that AN is indeed metabolized by brain microsomes to two principal metabolites, 16 mass units higher than AN, consistent with its conversion to a hydroxylated or epoxidized metabolite. In contrast to liver AN metabolism, however, P450 3A was only a minor contributor to its brain metabolism. Full characterization of the structure of these brain AN metabolites, as well as of their physiological significance, remains to be accomplished, but it is conceivable that they may represent a new important class of eicosanoids.

AN metabolism in other extrahepatic tissues may contribute to the peripheral actions of cannabinoids such as immunomodulation, alteration of intraocular pressure, and bronchodilation [21–23]. Metabolism in other extrahepatic tissues is yet to be elucidated but may prove to be clinically relevant. Furthermore, characterization of extrahepatic AN metabolism by other enzymes (cyclooxygenases, lipoxygenases) known to metabolize arachidonic acid may identify additional physiologically potent products.

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