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Preliminary Note

Metabolism of Progesterone in Mouse Brain

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Incubation of whole mouse brain homogenate with [^{3}H]progesterone resulted in two metabolites: the 5α-reduced product, 5α-pregnane-3,20-dione and another metabolite at a 3-fold greater yield. This differed from rat brain, which produced predominantly the 5α-reduced metabolite under the same conditions. Subcellular fractionation of mouse brain demonstrated a particulate location for the 5α-reduction of progesterone and a cytosolic location for the production of the unknown major metabolite. Treatment of this unknown metabolite with chromium trioxide resulted in a reconversion to progesterone, indicating the presence of a hydroxyl at position 3 or 20. Comparison of the chromatographic behaviour of the unknown metabolite with that of authentic progesterone derivatives suggested that this metabolite corresponds to 20-hydroxy-4-pregnene-3-one.

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INTRODUCTION

Anaesthetic [1, 2], anti-convulsant [3] and anti-anxiety [4] actions of progesterone are thought to be mediated by its 3α,5α-reduced metabolite 5(α-pregnane-3α-ol-20-one (allopregnanolone). This metabolite acts as a potent positive modulator of the neuronal chloride channels gated by the inhibitory transmitter γ-aminobutyric acid (GABA) at $GABA_A$ receptors in the central nervous system (CNS) [5–7]. Two enzymes catalyse the production of this metabolite [8], a rate-limiting progesterone 5α-reductase (5α-pregnane-3,20-dione: $NADP^+$ oxidoreductase, EC 1.3.1.30) and a 3α-hydroxysteroid dehydrogenase (3α-hydroxysteroid: $NAD(P)^+$ oxidoreductase, EC 1.1.1.50). To date, evidence to support 5α-reduction as the major route of progesterone metabolism in mammalian brain has come from studies on the rat [9–15]. During the course of experiments to establish assays of 5α-reductase activity in mouse brain, we found an alternative major route of progesterone metabolism [16] and now present a partial characterization of this pathway.

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MATERIALS AND METHODS

[1, 2, 6, 7, 16, 17-^{3}H]Progesterone (3.70 TBq/mmol) was purchased from Amersham International PLC. The reference steroids 4-pregnene-3,20-dione (progesterone), 5α-pregnane-3,20-dione (5α-dihydroprogesterone; 5α-DHP), 5β-pregnane-3,20-dione (5β-dihydroprogesterone; 5β-DHP), 5α-pregnane-3α-ol-20-one (allopregnanolone), 4-pregnene-20α-ol-3-one (20α-dihydroprogesterone; 20α-DHP) and 4-pregnene-20β-ol-3-one (20β-dihydroprogesterone; 20β-DHP) were obtained from Sigma Chemical Company, 4-pregnene-17α-ol-3,20-dione (17α-hydroxyprogesterone) from Aldrich Chemical Company and 4-pregnene-3β-ol-20-one (3β-hydroxyprogesterone) from Organon International. All other reagents and chemicals were purchased either from Sigma Chemical Company or BDH.

Adult male C57BL (Joint Animal House, U.C.L.) or To (Olac, U.K.) mice 20–40 g or adult male Wistar rats (Joint Animal House, U.C.L.) 250–300 g were used. Animals were kept in groups of 3–4 in standard plastic cages, under controlled conditions of temperature (20–22°C) with a 12 h light/dark cycle (lights on at 08.00 h) and had free access to food and water.

Mice were killed by cervical dislocation and rats by CO_2 inhalation prior to removal of whole brain. Samples were taken between 09.00–14.00 h and all

assays employed whole brain minus the olfactory lobes. Brains were homogenized with a Polytron (full speed, 10 s) in 10 vol of ice cold 0.1 M KH_2PO_4 buffer (pH 7.2) unless cytosolic and particulate fractions were to be prepared, in which case a Dounce homogenizer was used and brains homogenized in 10 vol of the same buffer containing 0.32 M sucrose. After centrifugation of the latter (100,000 *g*, 60 min), the clear supernatant was used as the cytosolic fraction and the pellet resuspended in homogenization buffer as the particulate fraction.

Samples (100 μl) of homogenate, cytosolic or particulate fractions were assayed in triplicate by incubation in 0.1 M KH_2PO_4 buffer (pH 7.2) containing 1 mM NADPH, 1 mM EDTA, 1 mM dithiothreitol and 10 nM [^{3}H]progesterone in a final total volume of 0.5 ml. After a 10 min preincubation at 37°C, reactions were initiated by the addition of [^{3}H]progesterone and samples incubated for a further 30 min at 37°C. The reaction was then stopped in an ice bath and steroids extracted with 2 ml chloroform:methanol (2:1, v/v) containing 100 μg each of unlabelled progesterone and 5α-DHP. Extracts were dried down in a desiccator under vacuum. Control incubations containing no tissue sample or boiled tissue samples were included in each experiment.

Substrate and product steroids were separated by TLC. Dried extracts were redissolved in 100 μl of chloroform and applied (80 μl) to 20 × 20 cm aluminium sheets pre-coated with silica gel (Merck). To maximize recovery of steroids, this last step was repeated for each sample. The appropriate reference steroids (50 μg) were applied to the outer lanes of each plate. Unless otherwise stated, the TLC plates were developed in solvent system C (cyclohexane:*n*-butylacetate 1:2, v/v). Reference steroids were visualized by exposure to iodine vapours followed by spraying with methanol (70%). Sample lanes were then cut into consecutive 1 cm bands, each of which was taken for measurement of radioactivity by liquid scintillation counting to generate radiochromatograms. Peaks of radioactivity along these chromatograms corresponding to substrate or product steroids were expressed as a percentage of the total radioactivity recovered from the incubation, which was found to be 82 ± 4% for mouse brain homogenates (Mean ± SEM; $n = 26$). Recovery of radioactivity from incubations of rat brain homogenate was 86 ± 8% (Mean ± SEM; $n = 4$). Radioactivity corresponding to product steroids in tissue sample lanes was adjusted by subtracting the activity at corresponding positions in boiled tissue sample control lanes and the corrected amounts of tissue sample product steroids expressed as fmol/mg protein. Protein concentrations of the tissue homogenates or fractions were determined using the dye-binding method of Bradford [17] with bovine serum albumin as the standard.

The identity of the major [^{3}H]progesterone metabolite formed in incubates of mouse brain cytosol was investigated after its purification by TLC, as described above. Prior to elution, the position of the metabolite was determined by generating radiochromatograms from the outer lanes of the plates. The peak of radioactivity corresponding to the major progesterone metabolite was then eluted from the inner lanes with chloroform: methanol (2:1, v/v). Purity of the eluted [^{3}H]-metabolite was assessed by subjecting samples (50,000 dpm) to further TLC. The identity of this [^{3}H]-metabolite was investigated by comparison of its mobility on TLC with that of a variety of unlabelled standard progesterone derivatives, after development in the following three solvent systems: (A) chloroform: ether (10:3, v/v), (B) hexane: ethylacetate (5:2, v/v) and (C) cyclohexane: *n*-butylacetate (1:2, v/v). The effect of oxidation with CrO_3 on the subsequent mobility of this [^{3}H]-metabolite on TLC in comparison to untreated [^{3}H]-metabolite or [^{3}H]progesterone was also investigated. For this modification, samples (100 μl–100,000 DPM) of [^{3}H]-metabolite were oxidised in 0.2 ml of a 1:1 (v/v) mixture of glacial acetic acid: aqueous CrO_3 (2%, w/v) for 2 h at room temperature. After addition of water to a total volume of 0.5 ml, the steroids were extracted and separated by TLC in solvent system C.

RESULTS

Incubation of mouse whole brain homogenates with [^{3}H]progesterone, followed by separation of the products by TLC in solvent system C, resulted in three main peaks of radioactivity corresponding to progesterone, 5α-DHP and another metabolite. The latter metabolite did not correspond with the 3α-reduced derivative of 5α-DHP, allopregnanolone. A typical radiochromatogram from this experiment is shown in Fig. 1. Amounts of [^{3}H]-steroid products formed and corresponding to [^{3}H]5α-DHP and the [^{3}H]-unidentified metabolite were calculated as 267 ± 64 and 765 ± 71 fmol/mg protein (Mean ± SEM; $n = 4$), respectively.

On fractionation of mouse brain homogenates, formation of [^{3}H]5α-DHP from [^{3}H] progesterone was found to be enriched in the particulate fraction, while formation of the unidentified [^{3}H]-metabolite was enriched in the cytosolic fraction. For the two fractionations performed, the mean amounts of [^{3}H]progesterone metabolites produced in particulate and cytosolic fractions, respectively, were as follows: [^{3}H]5α-DHP, 424.5 and 95.4 fmol/mg protein; [^{3}H]-unidentified metabolite, 337.5 and 4096.5 fmol/mg protein.

The unidentified mouse brain [^{3}H]progesterone metabolite was purified by TLC to facilitate subsequent characterization. On further TLC in solvent system C, the purified [^{3}H]-metabolite gave a single peak of radioactivity at an R_f of 0.29, in comparison with 0.41 for [^{3}H]progesterone. Further comparisons of the chromatographic behaviour of the purified [^{3}H]-

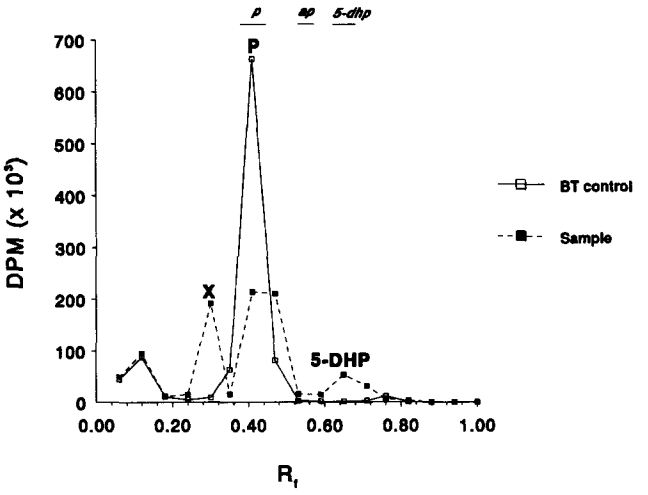

Fig. 1. Separation by TLC of the [^{3}H]progestins found after incubation of mouse brain homogenate (sample) or boiled tissue homogenate control (BT control) with [^{3}H]progesterone. The three main peaks correspond to [^{3}H]progesterone (P; $R_f = 0.42$), [^{3}H]5α-DHP (5-DHP; $R_f = 0.65$) and an unknown major [^{3}H]-metabolite (X; $R_f = 0.29$). Each point is the mean of three determinations and the experiment was repeated four times with comparable results. Horizontal bars indicate positions of the unlabelled reference steroids, progesterone (*p*), 5α-DHP (*5-dhp*) and allopregnanolone (*ap*).

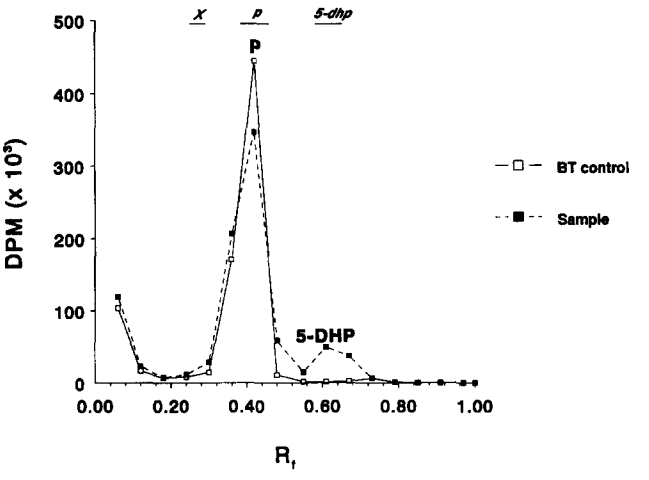

Fig. 2. Separation by TLC of the [^{3}H]progestins found after incubation of rat brain homogenate (sample) or boiled tissue control (BT control) with [^{3}H]progesterone. The two main peaks correspond to [^{3}H]progesterone (P; $R_f = 0.39$) and [^{3}H] 5α-DHP (5-DHP; $R_f = 0.64$). Each point represents the mean of three determinations and the experiment was repeated four times with comparable results. Horizontal bars indicate positions of the unlabelled reference steroids progesterone (*p*) and 5α-DHP (*5-dhp*) or of the major [^{3}H]progesterone metabolite formed by mouse brain homogenates (X; $R_f = 0.24$) and applied to neighbouring lanes on the plates.

metabolite with standard progesterone derivatives in three different solvent systems revealed R_f values similar to 20α- and 20β-DHP (Table 1). Treatment of the purified [^{3}H]-metabolite with CrO_3 resulted in a change in its chromatographic mobility in solvent system C ($R_f = 0.31$) to that of [^{3}H]progesterone ($R_f = 0.43$). All the R_f values given above were determined in triplicate and the experiments repeated twice with comparable results.

Table 1. A comparison of the R_f values of various progestins after separation by TLC using the solvent systems: (A) chloroform: ether (10:3, v/v), (B) hexane: ethylacetate (5:2, v/v or (C) cyclohexane: n-butylacetate (1:2, v/v)

	Solvent System		
	A	B	C
Progestin	R_f Value		
Progesterone	0.77	0.28	0.43
20α-dihydroprogesterone	0.46	0.15	0.29
20β-dihydroprogesterone	0.45	0.15	0.31
3β-hydroxyprogesterone	0.60	0.24	0.48
17α-hydroxyprogesterone	0.47	0.13	0.31
5α-dihydroprogesterone	0.84	0.33	0.62
5β-dihydroprogesterone	0.83	0.34	0.58
allopregnanolone	0.61	0.26	0.49
[^{3}H]-X	0.46	0.17	0.29

The compound [^{3}H]-X corresponds to the major mouse brain [^{3}H]progesterone metabolite purified by previous TLC in solvent system C, as described in Materials and Methods. The R_f values shown were determined in triplicate and the above separations repeated twice with comparable results.

In contrast to the results obtained on incubation of mouse whole brain homogenates, incubation of rat whole brain homogenate with [^{3}H]progesterone resulted in only two major peaks of radioactivity, corresponding to [^{3}H]progesterone and [^{3}H]5α-DHP. A typical radiochromatogram is shown in Fig. 2. The amount of [^{3}H]5α-DHP formed was calculated as 249 ± 27 (Mean ± SEM; $n = 4$) fmol/mg protein. The major [^{3}H]progesterone metabolite formed by mouse brain homogenates and purified as described above was included on adjacent lanes of each TLC plate and its migration is also illustrated in Fig. 2. The amount of this [^{3}H]-metabolite formed by incubates of rat brain was calculated to be 13.5 ± 3.6 fmol/mg protein (Mean ± SEM; $n = 4$).

DISCUSSION

Metabolism of progesterone in whole mouse brain homogenates was found to consist of two main pathways leading to the production of 5α-DHP and an unknown, more abundant metabolite, which accounted for at least a 3-fold greater conversion of progesterone than 5α-DHP. A different situation was observed with rat brain homogenates which, in agreement with previous reports [9-15], produced 5α-DHP as the major progesterone metabolite. Treatment of the unknown major progesterone metabolite produced by mouse brain with CrO_3 resulted in a reconversion to progesterone, indicating the presence of a hydroxyl group

at C_3 or C_{20}. However, comparison of the chromatographic behaviour of this metabolite with that of a variety of known progesterone derivatives revealed an R_f value similar to 20α- or β-DHP, not to 3β-hydroxyprogesterone. We were unable to obtain 3α-hydroxyprogesterone as a reference compound, but a TLC separation such as was observed in three different solvent systems between the [^{3}H]-metabolite and 3β-hydroxyprogesterone would not be expected between the α- and β-configurations of 3-hydroxyprogesterone. Thus, our investigations indicate reduction at C_{20} as a major route of progesterone metabolism in mouse brain.

Previous reports of progesterone metabolism in subhuman primate, rat and rabbit brain have described the presence of a 20α-hydroxysteroid dehydrogenase (now classified as estradiol-17β: NAD^+ 17-oxidoreductase, EC 1.1.1.62) in cytosolic fractions [13, 18, 19]. The 5α-reductase enzyme, by contrast, has been shown to have a particulate location in rat brain [10, 12, 15]. Our present observations in mouse brain are therefore consistent with earlier reports in showing cytosolic and particulate locations for the 20α-hydroxysteroid dehydrogenase (20α-OHSD) and 5α-reductase activities, respectively. Previous studies of 20-OHSD activity in mammalian brain have shown the 20-OH to be in the α-configuration [13, 18–20]; to the best of our knowledge, there are no reports of 20β-OHSD activity. We have not yet determined the configuration of the 20-OH of mouse brain 20-DHP, but have no reason to believe that the mouse will differ from other mammals in this respect.

Some reports already imply a physiological role for 20α-DHP in the regulation of neuroendocrine function. In the rabbit and the rat, for example, systemic or intracerebral administration of 20α-DHP has been shown to affect oestrus behaviour and gonadotrophin secretion [21–25]. Such effects are unlikely to be mediated by the intracellular progesterone receptor, at which binding of 20α-DHP is negligible [24, 26]. Unlike allopregnanolone, 20α-DHP would also be inactive as a $GABA_A$ receptor modulator [2, 27, 28]. Thus, the mode of action of 20α-DHP on the CNS awaits investigation, as does the extent to which the production of this metabolite, indicated by the present study of mouse brain, is typical of central progesterone metabolism in other mammals.

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