INDUCTION OF A SPECIFIC PROTEIN BY OESTRADIOL IN RAT PINEALS IN CULTURE

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1. Introduction

Several lines of evidence indicate that the pineal gland may be a target organ of steroid hormones. The rat pineal has been reported to take up and retain oestradiol both in vivo and in vitro, and to contain cytosol as well as nuclear components capable of binding oestradiol (reviewed [1,2]). Recent experiments have shown that the activity of hydroxyindole-*o*-methyltransferase (HIOMT), the enzyme involved in the last step of melatonin synthesis, is enhanced by oestradiol at the physiological concentration range, indicating the occurrence of a steroid-inducible reaction sequence in the pineal gland in culture [3]. The enhancement is abolished by inhibitors of protein synthesis, suggesting that de novo synthesis of a certain protein(s) is essential for the increase of this enzyme activity.

We report that oestradiol does in fact induce a protein species with app. mol. wt 97 000, which is distinct from the HIOMT protein per se, and similar in its electrophoretic mobility to the 'oestrogen-induced protein (IP)' reported for the rat uterus [4–6].

2. Materials and methods

2.1. *Labelling of pineal proteins*

Pineals from 4 ovariectomised rats were placed in a stationary position in a Petri dish containing 1.5 ml modified BGJ medium, and incubated at 37°C for 2 h in the atmosphere of 5% CO_2/95% air [3]. Two pineals each were then transferred to two separate dishes: the first dish contained, in 1.5 ml modified BGJ medium, L-[4,5-^{3}H]leucine (100 μCi), L-[4,5(*n*)-^{3}H]isoleucine (50 μCi) and 17β-oestradiol (15 nM); the second dish contained in the same medium L-[U-^{14}C]leucine (40 μCi), L-[U-^{14}C]isoleucine (24 μCi) and ethanol (10 μl) which was the vehicle of oestradiol. In some cases indicated (fig.1c), species of isotopes were exchanged between the two dishes, amino [^{14}C]-acids being used as precursors in the presence of oestradiol. After incubation for further 2 h at 37°C under 5% CO_2/95% air, 4 pineals from the two dishes were combined, homogenised in 400 μl ice-cold 1% Nonidet P-40 (Imperial Shell), centrifuged at 4°C for 60 min at 105 000 × *g*, and a 350 μl portion of the supernatant was subjected to gel electrophoresis.

2.2. *Labelling of uterine proteins*

This was carried out essentially as in [6]. Usually, the uterus from 1 immature rat was incubated at 37°C for 1 h in 1 ml Eagle's HeLa medium (Difco Laboratories), in the presence or absence of oestradiol (23 nM). Then, L-[4,5-^{3}H]leucine (20 μCi) was added to the oestradiol-treated group, and L-[U-^{14}C]leucine (5 μCi) to the control. After incubation at 37°C for 2 h, uteri from the two groups were combined, homogenised in 1.3 ml ice-cold 1% Nonidet P-40, centrifuged at 4°C for 50 min at 27 000 × *g* and 350 μl supernatant subjected to gel electrophoresis.

2.3. *Polyacrylamide gel electrophoresis*

This was carried out as in [7], with minor modifications. Each column consisted of the spacer gel (3%; 0.77 × 1.1 cm) and the running gel (7.5% or 5.0%; 0.77 × 9 cm). Buffers used were: 8.3 mM Tris–barbital/0.1% Nonidet P-40, pH 7.0 (reservoir buffer); 51 mM Tris–H_3PO_4/0.1% Nonidet P-40, pH 5.5 (spacer gel buffer); 71 mM Tris–HCl/0.1% Nonidet P-40, pH 7.5 (running gel buffer). Electrophoresis was carried out with the curent at 6 mA/tube until

the tracking dye (phenol red) migrated 7.1 cm from the origin (~ 2 h, 4°C). The gel was stained with 0.25% Coomassie brilliant blue in 50% ethanol/7.5% acetic acid, and destained in 30% ethanol/7.5% acetic acid, cut into 2 mm consecutive slices, and each slice was placed in a small vial in 0.5 ml Soluene-350 (Packard Instrument Co.), which was found to release all the radioactivity from the gel after shaking the vial at 37°C overnight. Radioactivity was measured in a Packard liquid scintillation spectrometer (model 3330) with 4 ml toluene-based scintillation fluid [3].

2.4. *Hydroxyindole-o-methyltransferase (HIOMT) activity*

After the electrophoresis at 4°C, the gel was cut into 2 mm consecutive slices, and each slice was ground in an all-glass homogeniser in 465 μl assay medium [3], and the homogeniser, per se, with pestle inside was incubated at 37°C for 60 min. HIOMT activity was determined as in [3,8].

3. Results and discussion

Incorporation of radioactive amino acids into the acid-insoluble material of the pineal homogenate is 96% inhibited by cycloheximide at 50 μg/ml. Oestradiol does not seem to affect the protein synthesis in terms of the total incorporated radioactivity (data not shown). However, electrophoretic fractionation of Nonidet P-40-soluble pineal proteins reveals that oestradiol does stimulate the synthesis of protein with a relative mobility of about 0.4 in the 7.5% gel (fig.1a). This is most clearly shown by a peak of induced protein which appears in this region when $^3H/^{14}C$ ratio is plotted against the relative electrophoretic mobility (fig.1b). When, contrary to the standard condition, amino [^{14}C]acids are included in the culture medium in the presence of oestradiol, and [^{3}H]amino acids in its absence, the peak of $^3H/^{14}C$ ratio reverses its polarity (fig.1c).

An attempt has been made to estimate the molecular weight of this induced protein, on the basis of relative mobilities obtained in gels of different concentrations [9,10]. Relative mobilities in the 7.5% gel (R_m 7.5) and in the 5.0% gel (R_m 5.0) of the induced protein and authentic proteins of known molecular weights were determined, and log [R_m 5.0/

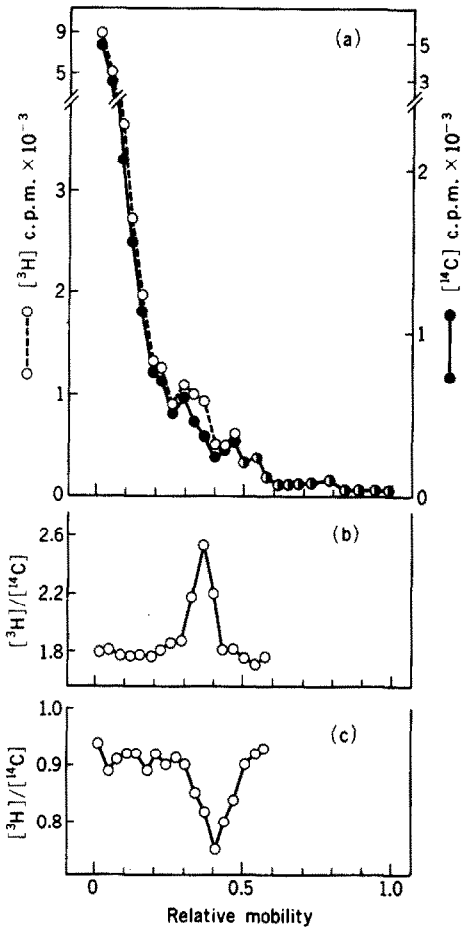

Fig.1. Polyacrylamide gel electrophoresis of Nonidet P-40-soluble pineal proteins. In (a) and (b), pineals labelled with [^{3}H]amino acids in the presence of 15 nM 17β-oestradiol, and pineals labelled with amino [^{14}C]acids in the absence of oestradiol were combined, homogenised in 1% Nonidet P-40, centrifuged and the supernatant subjected to electrophoresis on the 7.5% gel. In (c), pineals were labelled with amino [^{14}C]acids in the presence of oestradiol, and with [^{3}H]amino acids in its absence.

R_m 7.5] was plotted as a function of the molecular weight (fig.2); induced protein (horizontal arrow) is calculated to be app. mol. wt 97 000.

The possibility has been examined that the induced protein represents HIOMT itself (cf. [13]). Preliminary experiments have indicated that more than 95% HIOMT activity is solubilised from the pineal by 1% Nonidet P-40. Distribution of HIOMT activity after electrophoresis of the pineal supernatant in 1%

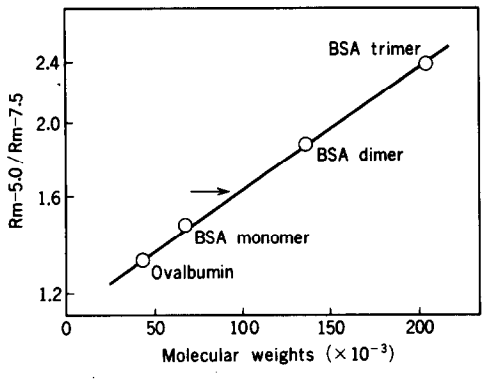

Fig.2. Determination of molecular weight of a pineal protein induced by oestradiol. Horizontal arrow indicates the position of the induced protein. Markers (mol. wt) included BSA (bovine serum albumin) trimer (204 000), dimer (136 000) and monomer (68 000), and ovalbumin (43 000).

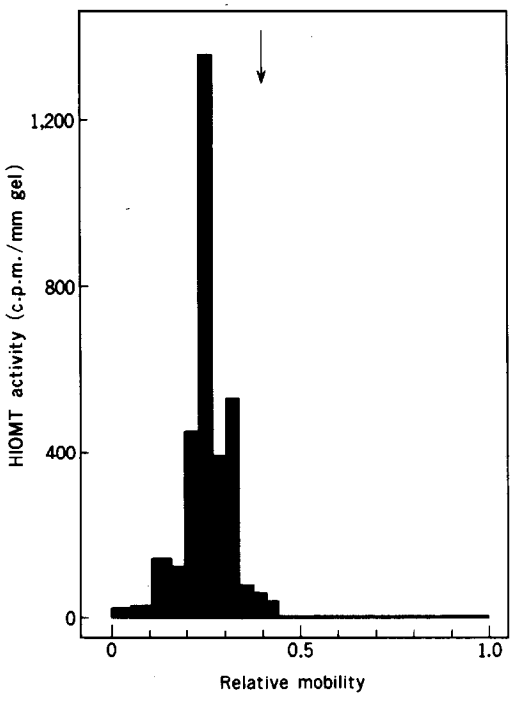

Fig.3. Distribution of hydroxyindole-*o*-methyltransferase (HIOMT) activity after its electrophoresis on 7.5% polyacrylamide gel. HIOMT was solubilised from the pineal by 1% Nonidet P-40. Vertical arrow indicates the position of the induced protein.

Nonidet P-40 is shown in fig.3. Relative mobility of HIOMT is obviously lower as compared with that of the induced protein, which almost negates the possible identity of these two proteins.

In spite of the observation that almost all the Nonidet P-40-soluble HIOMT activity enters into the gel (fig.3), recovery of the enzyme activity after its electrophoresis amounted only to 13% of that initially applied to the gel, which may be explained in several different ways.

(i) A simple grinding of the gel may not be sufficient to release all the HIOMT activity from the gel.

(ii) The catalytic site of HIOMT protein may be deteriorated during electrophoresis for 2 h at 4°C.

(iii) Accessory factor(s) required to elicit full activity of HIOMT may be dissociated during electrophoresis.

At present we do not know which possibility is most likely. Induced protein eluted from the gel, and added to the assay medium, failed to exert any stimulative action on the HIOMT activity (data not shown).

Next, we have examined the possibility whether or not the induced protein in the pineal resembles the 'oestrogen-induced protein (IP)' reported for the rat uterus [4–6]. In conformation of [6], we have

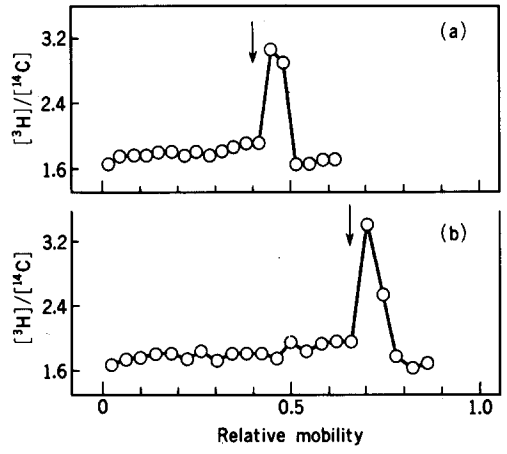

Fig.4. Induction of a uterine protein by oestradiol. The uterus labelled with L-[^{3}H]leucine in the presence of 23 nM oestradiol, and the uterus labelled with L-[^{14}C]leucine in the absence of oestradiol, were combined, homogenised in 1% Nonidet P-40, centrifuged, and the supernatant electrophoresed on 7.5% gel (a) and 5.0% gel (b). Arrows indicate the position for the pineal-induced protein.

observed de novo synthesis of protein induced by oestradiol in the uterus in vitro (fig.4). Relative mobilities of this uterine protein in the 7.5% and 5.0% gels are very much similar to, but not identical with those of the induced protein in the pineal (fig.4).

Induction of a specific protein(s) by oestradiol is currently evidenced in the uterus [4–6], in the hypothalamus [11] and in the pineal (present study), suggesting a common occurrence of this species of protein in the reaction sequence elicited by the steroid hormone. However, functional significance and chemical differences if any of the induced protein in various target organs await further elucidation.

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