Binding of ^3H -Progesterone by Isolated Rat Islets of Langerhans

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Summary. Islets of Langerhans isolated from normal female rats have been used in studies of ^3H -progesterone uptake by intact islet cells. The intracellular sites which are involved in binding of the hormones have been determined by subcellular fractionation and autoradiography. Uptake of ^3H -progesterone into islets occurred in a temperature and concentration dependent manner. The uptake increased rapidly in the first 30 min, and could be partially displaced by addition of excess unlabelled progesterone. ^3H -progesterone uptake was lowered by incubation of the islets in the absence of Ca^{++} , at 0° compared to 37°C , or to a much lesser extent when islet cyclic AMP levels were raised by addition of 3-isobutyl-1-methylxanthine. However, uptake was unaffected by prior treatment of the islets with neuraminidase or *p*-hydroxymercuribenzoate. Differential centrifugation of islets which had previously been incubated with ^3H -progesterone showed the highest specific activity of binding in the nuclei and debris fraction. Isolation of a purified nuclear fraction by sedimentation through sucrose solutions confirmed that binding was present in the nuclear component of this heterogeneous fraction, while autoradiographic studies suggested both nuclear and cytosolic localisation of ^3H -progesterone. In a separate series of experiments, evidence was obtained for the existence of saturable cytosolic binding of progesterone, and for movement of labelled hormone from the cytosol to the nuclear fraction.

It is suggested that, in the islets of Langerhans as in other target tissues, the action of progesterone involves penetration into the cells, binding to a cytosolic receptor protein, and subsequent transfer to the nucleus. The nuclear events which lead to subse-

quent alteration of rates of insulin secretion remain to be determined.

Key words: Progesterone binding, islet cell nuclei, electron microscopy, autoradiography, islets of Langerhans.

Administration of certain steroids to rats will induce hyperinsulinism and islet hypertrophy [1], and combination of progesterone and estradiol will effect changes somewhat similar to that observed in late pregnancy [2–5]. It has not been possible to observe short term direct effects of progesterone and estradiol on insulin secretion in vitro from isolated islets [2, 4]. However, enhanced insulin secretory responses were observed when islets were cultured in the presence of combinations of different concentrations of progesterone and estradiol for a period of 18 h [4]. The purpose of the present paper was to investigate the mode of action of progesterone in exerting these effects by examining some of the characteristics of uptake and binding of ^3H -progesterone to intact islets. Results of attempts to determine the intracellular localisation of binding sites by subcellular fractionation techniques as well as by the use of electron microscope radioautography are also reported.

Materials and Methods

Reagents and Animals

The Radiochemical Centre, Amersham, U.K., supplied (1,2,6,7-(n)- ^3H) progesterone of specific activity in the range 85–100 Ci/mmol. The following reagents were purchased from

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Sigma (London) Chemical Co. Ltd: progesterone, collagenase type I, albumin (crystalline) for protein determination, *p*-hydroxymercuribenzoate (Na salt), and neuraminidase. Collagenase type II was from Boehringer Corporation Ltd., Lewes, Sussex, U. K. Glass fibre discs GF/C, were from Whatman (Gallenkamp & Co. Ltd., London, U. K.). Female Sprague-Dawley rats weighing 250–300 g were used throughout. The rats were fed ad lib. a diet consisting of 20% protein, 54% carbohydrate and 3% fat. They had free access to water and were not fasted before experiments.

Methods

Isolation of Islets of Langerhans. Islets were prepared by collagenase digestion of the pancreas [22], using a mixture of equal parts of Sigma and Boehringer collagenases. In all experiments, islets were isolated in bicarbonate buffered medium [6] supplemented with 2 mmol/l glucose but without albumin.

^3H -Progesterone Binding to Intact Islets of Langerhans. Islets were distributed in groups of 20 into soda glass tubes (50×10 mm) containing a total volume of 0.4 ml of bicarbonate-buffered medium supplemented with 8 mmol/l glucose and 2.5 ng/ml of ^3H -progesterone. In some experiments other additions were made to the incubation medium as required, e. g. glucose (20 mmol/l), 3-isobutyl-1-methylxanthine (0.5 mmol/l), neuraminidase (25 $\mu\text{g}/\text{ml}$), in a final incubation volume of 0.4 ml. The tubes were then gassed with a mixture of 95% O_2 :5% CO_2 and incubated with shaking in a water bath at 37°C .

Free ^3H -progesterone was separated from islet bound steroid by filtration and washing the islets 5 times with cold 0.05 mol/l phosphate buffer, pH 7.4, through GF/C discs (2.5 cm diameter) placed on a Millipore filtration apparatus. The filter papers were then dried in 50×12 mm tubes at 70°C for 1 h before scintillant (toluene-triton X-100 – 2,5-diphenyloxazole 140:60:1 v/v/w) was added, and the radioactivity determined using a Beckman LS233 liquid scintillation counter. Similar counts were obtained if the islets were completely solubilized by addition of Soluene before counting. In each experiment, radioactive incubation medium alone was filtered through GF/C discs, and the number of counts obtained in these blanks, which was about 0.3% of the total applied, was subtracted from the test samples.

Preparation of Nuclei. The method employed is that originally described by Chauveau et al. [7], as modified by Knowler et al. [8]. Freshly isolated islets were incubated for 1 h in ^3H -progesterone (50 ng/ml), the incubation medium was removed and the islets washed in cold TKM buffer (0.05 mol/l Tris/HCl pH 7.5, containing 0.025 mol/l KCl and 0.005 mol/l MgCl_2) [9]. The islets were then homogenised in a glass homogeniser in a volume made up to 1.0 ml of TKM buffer containing 2.0 mol/l sucrose, and centrifuged for 1 h at 70,000 g. The floating debris and walls of the centrifuge tube were cleaned and the nuclear pellet resuspended in 1 ml of TKM buffer with 1.7 mol/l sucrose. The suspension was filtered through a 100 mesh grid [8], layered over 2 ml of 1.8 mol/l sucrose in TKM buffer, and centrifuged for 20 min at 15,000 g. Filtering through the grid leads to substantial losses of material, but gives a very much purer nuclear preparation.

Subcellular Fractionation of Islets. Groups of 200 islets were incubated in 1.5 ml of bicarbonate buffered medium containing 8 mmol/l glucose and 25 ng/ml ^3H -progesterone for 1 h at 37°C ; after removal of the incubation medium, the islets were washed several times in similar medium to remove excess radioactivity. Subcellular fractions were prepared from the islets as described in detail by Howell et al. [10]. Islets were homogenised in a glass

homogeniser in 0.5 ml of 0.01 mol/l Tris buffer, pH 7.4, containing 0.3 mol/l sucrose. This and all subsequent steps were carried out at 0°C . The homogenate was prepared by differential centrifugation into a nuclei and debris fraction (600 g for 5 min), a mixed mitochondrial, microsomal and secretory granule fraction (24,000 g for 60 min) and a supernatant. The two particulate fractions were resuspended in the homogenisation buffer and aliquots of each were used to determine the total amounts of radioactivity present, the radioactivity which was bound, and the protein content [11] of the fraction.

Binding Studies Using a 105,000 g Supernatant Protein Preparation from Islets. Groups of 200 islets were homogenised in a glass homogeniser in 1.0 ml of Gey and Gey buffer [6] and this homogenate was centrifuged at 0°C at 105,000 g for 1 h. Aliquots (100 μl) of the supernatant were incubated with 50 μl of 25 ng/ml ^3H -progesterone and 50 μl buffer [6] at 37°C with shaking for 1 h, and the progesterone bound to the supernatant protein was separated by filtration as described above. In some experiments, the quantities of supernatant or of radioactivity were varied as described in the Results.

Electron Microscopy. Islets which had been incubated with ^3H -progesterone (50 ng/ml) for 1 or 2 h at 37°C , or alternatively isolated nuclear fractions, were fixed with 3% glutaraldehyde in 0.1 mol/l phosphate buffer pH 7.4, post-fixed with OsO_4 in similar buffer, dehydrated and embedded by a standard procedure. In autoradiographic experiments involving islets which had been pre-labelled ultrathin sections were coated with Ilford L4 emulsion (Ilford Ltd., Ilford, U. K) and exposed for periods of 6–20 weeks before development in Kodak K 19 developer; the procedures involved have been described previously [24]. Thin sections and autoradiographs were stained with a saturated solution of uranyl acetate in 50% ethanol before examination in a Jeol 100S electron microscope.

Results

^3H -progesterone is taken up by rat islets of Langerhans, and equilibrium concentrations were reached after 30 min (Fig. 1A). The uptake was proportional to the concentration of progesterone in the incubation medium (Fig. 1B) and could be displaced by 46% in the conditions studied, by addition of unlabelled progesterone (Fig. 1C).

The accumulation of progesterone within islet cells could be altered under certain incubation conditions (Table 1). Substances which alter the composition of the plasma membrane, e. g. *p*-hydroxymercuribenzoate or neuraminidase [12] did not affect progesterone uptake, whereas low temperature (0°C) or the absence of extracellular Ca^{++} significantly inhibited accumulation (Table 1). Incubation with 3-isobutyl-1-methylxanthine, which raised cyclic AMP levels, tended to inhibit progesterone uptake, though the effect was not statistically significant (Table 1).

Table 2 shows the distribution of radioactivity in the subcellular fractions of islets which were pre-labelled by incubation in ^3H -progesterone for 1 h. Although radioactivity was present in all the frac-

Fig. 1. Isolated intact islets of Langerhans were incubated in groups of 20 in 0.4 ml of buffer containing ³H-progesterone, for different periods of time (A) or for 60 min (B & C). The radioactivity taken up is plotted on the vertical axis: in B & C different amounts of labelled or unlabelled progesterone respectively were present in the incubation medium. Each of the points is the mean $\pm$ SEM of 5 (A) or 10 observations (B & C)

Table 1. Effect of different incubation conditions on ³H-progesterone uptake by isolated islets. The results are expressed as percentage of control (mean $\pm$ SEM) of 10–15 observations

Glucose (mmol/l)	Incubation conditions Addition to medium	Ca ⁺⁺ (mmol/l)	Temperature (°C)	Percentage of control: [³ H-progesterone uptake in 2 mmol/l glucose]
2	—	2	37	100
2	IBMX, 0.5 mmol/l	2	37	85 $\pm$ 7.1
2	<i>p</i> -OH-MB, 0.05 mmol/l	2	37	104 $\pm$ 5.0
2	Neuraminidase, 25 μ g/ml	2	37	102 $\pm$ 7.2
20	—	2	37	98 $\pm$ 9.0
20	IBMX, 0.5 mmol/l	2	37	96.5 $\pm$ 10.0
2	—	0	37	73.1 $\pm$ 6.6 ^a
2	—	2	0	55.2 $\pm$ 3.7 ^a

The abbreviations used are: IBMX = 3-isobutyl-1-methylxanthine; *p*-OH-MB = *p*-hydroxymercuribenzoate

^a indicates results significantly different from control, $p < 0.01$ by 't' test

tions, the nuclei and debris fraction showed the highest accumulation in terms of CPM/ μ g protein. ³H-progesterone binding to isolated nuclei prepared by sedimentation through sucrose solutions (as in Fig. 2) was obtained. The recovery of protein in this purified nuclear fraction was about 5% of the total in the homogenate making detailed studies of binding difficult. However, nuclei isolated from islets which had been incubated with ³H-progesterone at 0°C contained significantly less progesterone (62 $\pm$ 5%) than those incubated at 37°C (100%) in the same experiment (mean $\pm$ SEM of 3 experiments).

Electron Microscope Autoradiography. Preliminary experiments suggested that 70% of ³H-labelled progesterone present in the islets was extracted from the

Table 2. Distribution of ³H-progesterone binding in subcellular fractions of rat islets. Groups of 200 islets were incubated for 1 h at 37°C in 1.5 ml of bicarbonate buffered medium containing 8 mmol/l glucose and 25 ng/ml ³H-progesterone. Islets were homogenised and subcellular fractions prepared by differential centrifugation [10]. The results (means $\pm$ SEM) of 4 experiments are expressed as CPM/ μ g protein

Fraction	³ H-progesterone bound CPM/ μ g protein
Islet homogenate	227 $\pm$ 34
Subcellular fractions:-	
(a) Nuclei and debris (600 g $\times$ 5 min)	532 $\pm$ 95
(b) Mitochondria + granules + microsomes (24,000 g $\times$ 60 min)	204 $\pm$ 22
(c) Supernatant	28 $\pm$ 4

Fig. 2. Electron micrograph of islet cell nuclei isolated by the density gradient centrifugation procedure outlined in the text. Uranyl acetate. $\times 8,200$

Fig. 3. Electron micrograph autoradiograph of B cell in an islet which had been incubated with 50 ng/ml ^3H -progesterone for 2 h prior to fixation. Silver grains appear to be present in both cytoplasmic and nuclear areas. Uranyl acetate. $\times 8,550$

tissue during the fixation and dehydration procedures employed in specimen preparation for electron microscopy. Autoradiographic localisation of that fraction of the label which remained showed the presence of silver grains over nuclei and in the cytoplasm, but not specifically associated with storage granules, mitochondria or plasma membranes (Fig. 3).

Intracellular Binding of ^3H -progesterone. The binding of ^3H -progesterone to cytoplasmic islet proteins and the relationship between this binding and the transfer of radioactivity to the nuclear fraction was studied (Figs. 4 and 5). ^3H -progesterone binding to a high speed (105,000 g for 60 min) supernatant fraction of islets was saturable (Fig. 4B), unlike uptake into intact islets (cf Fig. 1B). Binding proceeded over a

1 h period (Fig. 4A) but fell later on, 4 h up to 24 h, and was partially displaceable on subsequent addition of unlabelled progesterone to the incubation medium (Fig. 4C). A Scatchard plot over the range of concentrations of ^3H -progesterone (free hormone) of 1.25–25 ng/ml indicated a K_d of 1.2×10^{-8} mol/l for total binding. In order to determine whether progesterone binding in the cytoplasm may be followed by a temperature dependent transfer to the nuclear fraction, islets were incubated in ^3H -progesterone for 1 h at either 37°C or 0°C . The radioactive medium was then removed and the islets were re-incubated for a period of 1 h in buffer alone at either 37°C or 0°C . After incubation of islets with ^3H -progesterone at 37°C for 1 h radioactivity was distributed approximately equally between the cytoplasmic and nuclear

Fig. 4. A high speed supernatant fraction (105,000 g for 60 min) was prepared from 200 islets of Langerhans homogenised in 1 ml of bicarbonate buffer. 100 μl of the supernatant were incubated with 50 μl of ^3H -progesterone (50 ng/ml) + 50 μl buffer for different time periods (A) or for 1 h (B & C). In B & C different amounts of radioactive or non-radioactive progesterone were added to the incubation medium. In A, the results were plotted as percentage of maximal binding. Each point on each graph is the mean $\pm$ SEM of 8–12 observations

fractions, while after labelling at 0°C for a similar period ^3H -progesterone accumulated in the cytoplasmic fraction. Following a 1 h period of incubation at 37°C after a 0°C incubation, 75% of the label was transferred to the nuclear fraction, whereas if the second incubation was performed at 0°C only 25% of the radioactivity appeared in the nuclear fraction (Fig. 5).

Discussion

According to Bartholomeusz et al. [18] average daily plasma progesterone concentrations in non-pregnant rats may be around 5 ng/ml, rising to 50 ng/ml or more at the start of the third week of pregnancy; the progesterone level varies considerably during the course of a day. In the present study we have used progesterone concentrations which fall into that definition of the normal physiological range, i.e. 2.5 ng/ml, or 25–50 ng/ml when incorporation of a large amount of label was desired. In a 1 h incubation approximately 5% of progesterone was taken up into islets and there was no degradation of added steroid as determined by extraction from the incubation medium and chromatography on thin layer strips (results not shown).

Uptake of labelled hormone by islets was found to be temperature and concentration dependent, and to require at least 30 min to reach equilibrium; this is followed by a longer period of binding to cytosolic and nuclear sites [20] and is consistent with the observed lack of a direct effect of progesterone on islet insulin release during a 60 or 90 min period of

Fig. 5. Groups of 200 islets were incubated for 1 h in 50 ng/ml ^3H -progesterone, followed by a further 1 h in bicarbonate-buffered medium alone in the temperature conditions shown above. After 2 h the islets were homogenised. The radioactivity and protein content of the 105,000 g for 60 min supernatant and pellet were estimated. The results from 4 individual experiments with small variations are expressed as a ratio

incubation [2, 4]. The binding to intact islets showed a complex kinetic pattern, as would be expected from the presence of multiple binding sites which were observed subsequently. Detailed analysis of the kinetics of binding was therefore only performed on

the supernatant fractions and not on intact islets; a saturable binding species was found in the cytosol with binding characteristics similar to those found in other systems [13–16, 19, 20]. The degree of binding to the cytoplasmic receptor was temperature dependent (Fig. 5) as was the transfer of binding from the cytoplasmic to nuclear fractions [13, 17].

The localisation of labelled steroids by autoradiographic technique is rendered difficult by their solubility in the fixatives and other solvents which are required for processing the material for electron microscopy. Thus true localisation can only be achieved by use of 'diffusible compound' techniques on frozen sections of unfixed tissue. This has been performed successfully at the light microscope level by Stumpf and Sar in a variety of tissues; their studies have shown both nuclear and cytoplasmic binding according to the nature of the tissue and the conditions which were studied [21]. We have found, using islets of Langerhans, that fixation and dehydration required for electron microscopy resulted in partial but not complete loss of radioactivity, some 30% of the total radioactivity being retained in the tissue. We therefore attempted to localise this remaining fraction of bound material by electron microscope autoradiography and found that it was present in the nuclear and to a lesser extent in cytoplasmic areas, a result which is consistent with the biochemical data.

Although we have not investigated the post nuclear binding stages of progesterone action in islets, the presence of cytosolic and nuclear binding and the apparent absence in the autoradiographs of plasma membrane binding would seem to reduce the possibility that the effects of progesterone on insulin release are mediated via direct effects on adenylate cyclase. In other experiments no changes in activity of this enzyme have been detected in islets taken from progesterone injected rats, or in islets which have been cultured with progesterone, and no direct effects of steroids on adenylate cyclase activity *in vitro* have been found [4]. The recent reports of raised cyclic AMP levels in islets from progesterone injected rats may therefore result from other actions of the hormone [23].

The evidence presented here suggests that progesterone, at the concentrations present in normal plasma, can penetrate the cells of the islets of Langerhans. This is followed by binding to cytosolic receptor proteins and by a temperature-dependent transfer of the steroid from the cytoplasmic to nuclear fraction. These features are common to the actions of a wide variety of steroid hormones in different tissues [13–17, 19–21]. The mechanism by which nuclear binding leads to long term alterations in the regulation of insulin release remains to be determined.

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Note added in proof. Addition of cortisol (1 μM) to incubations of islet supernatant with ^3H -progesterone resulted in a slight reduction in ^3H -progesterone binding indicating that some non-specific binding of ^3H -progesterone in islet supernatant could be detected.