Diethylpyrocarbonate, a Histidine Selective Reagent, Causes Structural Alteration of Rat Ovarian LH/hCG Receptor

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Abstract. Treatment of rat ovarian membrane-bound and Triton X-100 solubilized LH/hCG receptor with a histidine-specific reagent diethylpyrocarbonate (DEPC) resulted in inactivation of the ability of the receptor to bind hCG The partial reversibility of this inhibition by hydroxylamine demonstrated that histidine residues are involved in hCG-receptor binding Fluorescence quenching experiments indicated that DEPC did not change the accessibility of fluorophores for acrylamide Alterations of quenching rate generally suggest exposure of tryptophanyl residues Modification of histidyl residues was connected with an alteration of the physical state of ovarian membranes Membrane lipid rigidity was decreased after DEPC reaction Thermal perturbation techniques were used to monitor structural changes in the receptor due to the action of DEPC on membranes Heat inactivation of hCG-binding sites demonstrated that there was a significant destabilization of the LH/hCG receptor structure when the membranes were treated with DEPC Thermal destabilization produced by 5 mmol/l DEPC caused a decrease in T_{50} values by about 12°C These results suggest that histidine residues are located at the binding sites of the receptor and that they are also involved in alterations of membrane proteins the structural integrity of which secondarily influences the accessibility of the LH/hCG receptor

Key words: Diethylpyrocarbonate LH/hCG receptors Thermal inactivation Fluorescence polarization

Introduction

Gonadotropin receptors, which are responsible for transmembrane communication

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and numerous functional interactions within the plane of the membrane are embedded in the lipid bilayer. The cell receptor for LH/hCG is though to be an integral protein containing seven transmembrane segments (Segaloff and Ascoli 1993). The immediate response of target cells to the binding of LH/hCG is an increase in adenylylcyclase activity mediated by G-proteins. The subsequent cAMP generation results in increased steroid hormone synthesis. The responsiveness of tissues to gonadotropins was defined by the numbers of LH/hCG receptors and/or their functional activity. Because of the lack of knowledge about the receptor structure it is difficult to study the molecular mechanisms of the hormone-receptor interaction. Cloning of the cDNA for the LH/hCG receptor has shown that the amino terminal domain which is sufficient for the binding of the hormone is composed of a repeating leucine rich repeat motif (Braun et al. 1991). However mutagenesis along with crystallographic experiments of hormone receptor complexes will help establishing the specific amino acids essential for ligand binding. An alternative method is to use chemical modification of amino acid residues with protein-modifying reagents. For that purpose we studied the inhibitory effect of diethylpyrocarbonate on hCG binding to the receptor, a compound that has been previously used to study the role of histidine in enzymes and hormone binding proteins (Kolena and Šebokova 1987, Hollis and Strange 1992). Although diethylpyrocarbonate can react with a large number of nucleophilic amino acid residues, it shows a good selectivity for histidine at neutral pH. Histidine residues of the LH/hCG receptor have been implicated as necessary for the hormone binding (Kolena and Šebokova 1987). From these studies however, it is not clear if the inhibition of binding was due to a modification of the binding sites or to changes in membrane protein which can affect the receptor recognition phenomena. In the present work structure-functional relationships of the LH/hCG receptor was studied by intrinsic fluorescence and thermal perturbation techniques after diethylpyrocarbonate inactivation of the ability of the receptor to bind hCG.

Materials and Methods

Materials

Purified hCG (CR 123, 12,780 IU mg^{-1}) was generously supplied by NIAMDD, NIH, Bethesda. $Na^{125}I$ was purchased from the Radiochemical Center, Amersham. Pregnant mare's serum gonadotropin (PMSG), hCG (Praedyn) were from Spofa, Prague. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Serva (Heidelberg, Germany), and all other chemicals were from Sigma (St. Louis, MO, USA).

Methods

Luteinized ovaries were produced in 25-day-old rats (Wistar strain) by sc. administration of 50 IU PMSG followed by 30 IU hCG 56 h later (Kolena et al. 1990).

Homogenates of ovaries (100 mg ml^{-1}) in icecold buffer A (25 mmol/l NaH_2PO_4, 1 mmol/l EDTA, 40 mmol/l NaCl, pH 7 4) were filtered through six layers of surgical gauze, centrifuged at $1000 \times g$ for 15 min, and the supernatant was further centrifuged at $20,000 \times g$ for 30 min The final membrane preparations were resuspended in the same buffer (Kolena et al 1986) Soluble LH/hCG receptors were prepared by extraction of the particulate binding fraction with 1% Triton X-100 for 30 min at 4 °C

Chemical modification of membrane by diethylpyrocarbonate (DEPC) was carried out for 30 min at 24 °C in buffer A, pH 7 0 DEPC was dissolved in ethanol The concentration of ethanol was kept below 5% Membranes were then centrifuged and washed twice as described above

Thermal perturbation techniques were used to probe structural features of the LH/hCG receptor Aliquots of membrane-bound receptor were heat inactivated in water bath at a constant temperature of 50 °C or by raising the temperature at a linear rate of about 1 °C/3 min Membrane preparations were withdrawn at designated temperatures and placed on ice until the determination of binding activity (Kolena et al 1994)

In hCG binding assay, 0 1 ml aliquots of ovarian membranes were incubated for 16 h at 20 °C with 0 1 ml buffer A + 1 mg ml^{-1} BSA with or without 100-fold excess of unlabeled hCG and 0 1 ml [^{125}I]hCG (1 1 5 ng, spec act about 2 3 TBq g^{-1}) After incubation and centrifugation the membrane pellets were washed twice with buffer A (Kolena et al 1986) The hormone-receptor complex in soluble receptor was precipitated twice with polyethylene glycol (Kolena and Šeboková 1987) The results are expressed as [^{125}I]hCG specific binding per mg protein (Lowry et al 1951)

Fluorescence polarization of DPH probe was measured by a Perkin-Elmer LS-5 luminescence spectrometer equipped with a circulation bath to maintain the sample temperature at 25 °C A solution of 2 mmol/l DPH in tetrahydrofuran was dispersed by 1000-fold agitative dilution in buffer A, pH 7 4 Ovarian membranes (100 μg protein) were incubated at 25 °C for 1 h with 2 ml of DPH in the above buffer The fluorescence polarization was computed by equation

$$P = \frac{I_{vv} - I_{vh}(I_{hv}/I_{hh})}{I_{vv} + I_{vh}(I_{hv}/I_{hh})}$$

where I_{vv} and I_{vh} are fluorescence intensities detected through a polarizer oriented parallelly and perpendicularly to the direction of vertical polarized light I_{hv}/I_{hh} represents the ratio when excitation is polarized horizontally and emission is observed through the analyzer oriented perpendicularly and parallelly, respectively (Kolena et al 1986)

Quenching studies were carried out at 23 °C by adding small amounts of 5 mol/l acrylamide in buffer A, pH 7 4 The intrinsic fluorescence intensity was measured as

a function of quencher concentration at a fixed emission wavelength of 416 nm The excitation wavelength of 280 nm was used The Stern Volmer quenching constant K_{sv}, was calculated according to the Stern-Volmer equation $F_0/F = 1 + K_{sv}$ [Q] where F_0 is the fluorescence of the unquenched fluorophore and F is the fluorescence at quencher concentration [Q] (Efting and Ghiron 1976 Kolena et al 1995) The least-squares method was used to calculate the K_{sv} constant

Data were analyzed by ANOVA and Bonferroni post test Values were considered statistically significant at $p < 0.05$ The results were confirmed in 2 3 independent experiments

Results

Incubation of ovarian membranes with millimolar concentrations of DEPC at pH 7 resulted in a concentration dependent decrease in hCG specific binding activity (Fig 1) After 30 min of reaction half maximal inhibition of [^{125}I]hCG binding was observed at approximately 1 5 mmol/l DEPC, and more than 90% inhibition of binding was achieved at 10 mmol/l reagent Similar observations were made after the treatment of Triton X-100 soluble receptor with DEPC However the

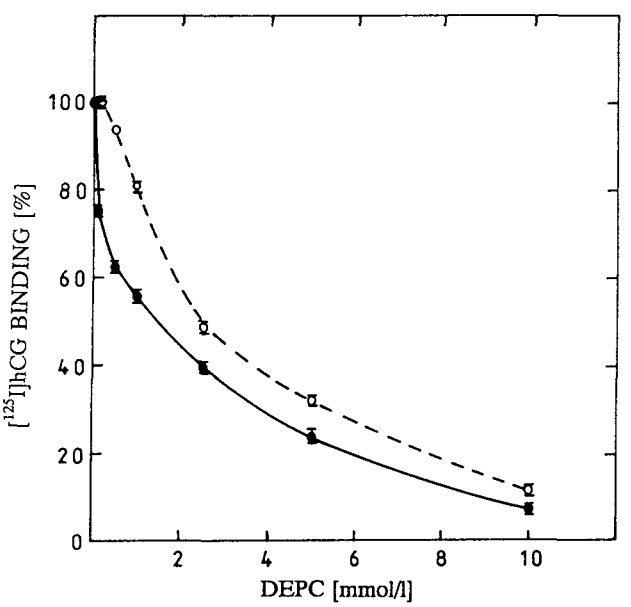

Figure 1. Concentration dependent inactivation of [^{125}I]hCG binding to membrane bound (full line) and Triton X-100 solubilized (dashed line) receptor by diethylpyrocarbonate (DEPC) Ovarian membranes were treated with DEPC for 30 min at 24 °C in buffer A pH 7 and assayed for hCG binding as described in the text Each point represents mean ± S E of three estimations

extent of loss of hCG binding was slightly less than in membrane-bound receptor. Modification with DEPC was carried out using conditions expected to result in a selective attack on histidine residue in protein (Pilch 1982). Other amino acids (cysteine, tyrosine, tryptophan and lysine) can also be changed by DEPC though less specifically. In order to examine whether the inactivation of the receptor was due to modification of histidine residue the receptor was treated with hydroxylamine. Hydroxylamine is a strong nucleophile that preferentially displaces the carbethoxy group from modified histidine and tyrosine residues, but not from cysteine and lysine residues (Miles 1977). Table 1 shows that hydroxylamine partially restored the binding of hCG to membrane ovarian receptor after inhibition with DEPC ($p < 0.01$).

Table 1. Reversal by hydroxylamine of membrane LH/hCG receptors inactivation by diethylpyrocarbonate. Ovarian membranes were incubated with 2.5 mmol/l DEPC and washed membranes were treated with hydoxylamine for 30 min at 21°C. Data are means ± S.E. of three estimations.

	Addition	$[^{125}I]$hCG bound (fmol/mg protein)	% of control
0	0	211 ± 2.0	100
0	NH_2OH (0.1 mol/l)	218 ± 3.5	103
DEPC	0	51 ± 6.6	24
DEPC	NH_2OH (0.2 mol/l)	73 ± 1.0	35
DEPC	NH_2OH (0.1 mol/l)	91 ± 6.2	43

Further experiments were performed to determine whether the changes in the LH/hCG receptor are linked with an alteration of the physical state of membranes preincubated with DEPC. As shown in Fig. 2 ovarian membrane rigidity as determined by fluorescence polarization of DPH decreases in membranes treated with DEPC. Information concerning the exposure of tryptophan residues can be obtained from results of the fluorescence quenching behavior of proteins. We used acrylamide, a neutral dynamic quencher, to found out whether DEPC modifies the quenching of protein fluorescence. An increase in quenching rate generally suggests an increase in the proximity of quencher molecules to the fluorophore. The Stern-Volmer constants (K_{sv}) determined from the Stern-Volmer plots for control and 0.1 and 1 mmol/l DEPC treated membranes were found to be 4.7 l/mol vs. 5.7 and 5.2 l/mol respectively, indicating that DEPC does not change the accessibility of fluorophores (tryptophan residues) for acrylamide (Fig. 2). The emission maximum

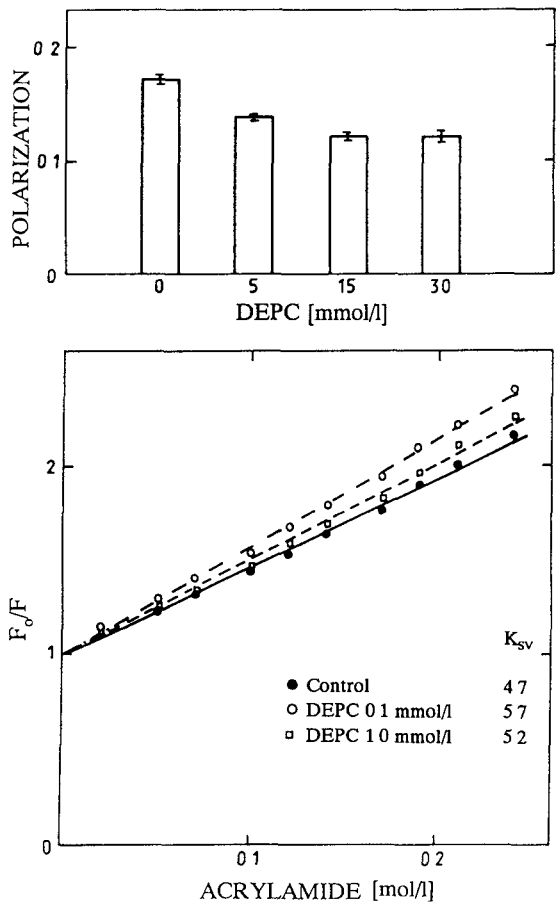

Figure 2. Effect of different concentrations of DEPC on fluorescence polarization of DPH probe (upper panel) and Stern Volmer plots with constants (K_{sv}) of acrylamide quenching (lower panel) for ovarian membranes. Experiments were done as described in the legend to Fig. 1.

of 416 nm for control membranes was not changed in membranes preincubated with DEPC (data not shown).

Thermal inactivation of the receptor is a rapid process. During the incubation of ovarian membranes treated with 1.5 mmol/l DEPC at a constant temperature of 50 °C the damage of binding sites was appreciably manifested after 5 min. The thermal inactivation process represents a temperature-dependent loss of LH/hCG binding sites that can be expressed in term of their T_{50} value i.e. the temperature at which 50% of initial binding capacity remains (Aitigues et al. 1989). The T_{50} value

of about 50 °C in control membranes decreased to 45 °C and 38 °C in membranes treated with 2 5 and 5 mmol/l DEPC, respectively (Fig 3)

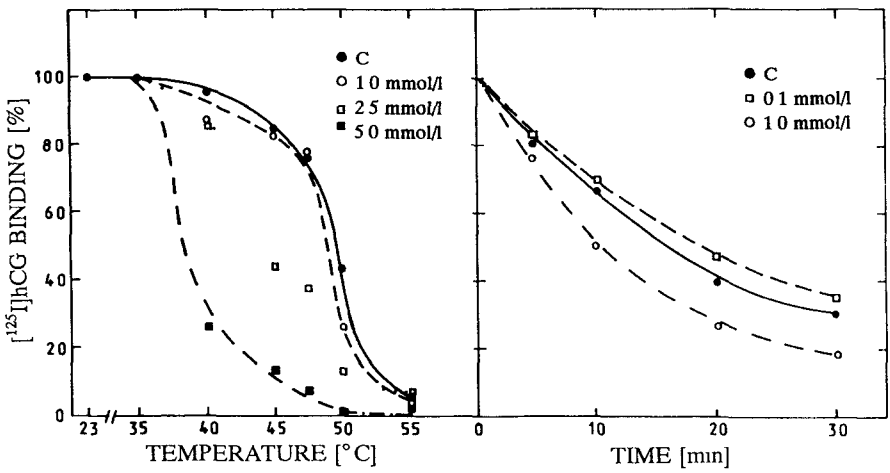

Figure 3. Thermal destabilization of ovarian membrane LH/hCG receptor with DEPC Ovarian membranes preincubated without or with different concentrations of DEPC were heat inactivated at increasing temperatures at a linear rate of 1 °C/3 min or at a constant temperature of 50 °C Means of two estimations are shown

Discussion

In this report using chemical modification of amino acids residues with diethylpyro carbonate we supply evidence for the importance of histidine at the ligand binding site of LH/hCG receptors DEPC reacts with the nucleophilic imidazole group of histidine ($pKa \approx 6$) the phenolic group of tyrosine ($pKa \approx 10$) the thiol group of cysteine ($pKa \approx 8\ 2$), the epsilon amino group of lysine ($pKa \approx 10\ 5$), and with amino terminal groups ($pKa > 9$) Considering these pKa values it would be expected that at pH 7 or below the imidazole group of histidine is the nucleophile on the protein that preferentially reacts with DEPC At this pH approximately 50% of the imidazole groups would be deprotonated as compared to approx 1% or less of other amino acids groups Additional information on the DEPC reacting nucleophile can be obtained from its incubation with hydroxylamine Hydroxylamine is known to remove carbethoxy group primarily from modified histidine residues (Miles 1977) When DEPC-inactivated LH/hCG receptor was treated with hydroxylamine, the binding activity was partly restored The incomplete reversal of hCG binding by hydroxylamine may be due to modification of other residues of

the receptor protein that are necessary for hCG binding or to the formation of dicarbethoxyhistidyl residues by the action of DEPC and to the addition of hydroxylamine resulting in ring cleavage thus causing irreversible modification. Since DEPC inhibition could be reversed by hydroxylamine, the modified amino acid residue crucial for ligand binding to the LH/hCG receptor is probably histidine. In addition to preferential carbetoxylation of the histidine residue, DEPC reacts with other amino acids residues such as lysine, cysteine, tryptophan and with an unusually reactive tyrosine residue. Since 20 mmol/l concentrations of acetic anhydride, which mainly reacts with lysyl residues, had no effect on hCG binding this amino acid is not likely to be important for the binding interaction. In contrast, tyrosyl residue may be involved in LH/hCG receptor binding sites because N-acetylimidazole and 2,4-dinitrofluorobenzene which are tyrosine-preferring reagents (Chatterjee 1971) inhibited the binding of hCG to the receptor (Kolena and Šebokova 1987). The role of tryptophan residues in LH/hCG binding sites is unclear. Quenching experiments indicated that DEPC did not change the accessibility of fluorophores for acrylamide. Alterations of quenching rate generally suggest exposure of tryptophanyl residues. Modification of tryptophanyl residues by DEPC can probably be ruled out because no change was observed in the spectral properties of membrane emission fluorescence.

Experiments with Triton X-100 soluble receptor showed that histidine residues are directly involved in hormone-receptor interaction, i. e., at the hCG binding sites of the receptor. However, it cannot be ruled out that DEPC reaction arises at a site distant from the binding sites. Data showed that modification of histidyl residues are connected with an alteration of the physical state of ovarian membranes. Membrane lipid rigidity decreased after DEPC reaction. The ordering of the membrane environment in which the LH/hCG receptor is embedded can affect the accessibility of the receptor. A positive correlation between the elevation of membrane rigidity and the accessibility of LH/hCG receptors was found in rat ovarian and testicular membranes (Kolena et al. 1990, Kolena and Kasal 1989).

Studies of heat inactivation of hCG binding sites were carried out to monitor structural alterations of the LH/hCG receptor. These techniques are highly selective to monitor general structural changes of the receptor protein (Artigues et al. 1989, Kolena et al. 1995). Thermal inactivation of hCG-binding sites suggested that there was destabilization of the LH/hCG receptor structure upon subjecting membranes to DEPC reaction. Thermal destabilization produced by 5 mmol/l DEPC caused a decrease in T_{50} values by about 12 °C. Modification of the LH/hCG receptor by DEPC suggests that histidyl residues are located at or close to the hCG binding sites, as well as that perturbations of the membrane at a site distant from the binding sites elicits an alteration of structure-related functional properties of the receptor.

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