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FUNCTIONAL CHARACTERIZATION OF THE CLONED HUMAN ACTH RECEPTOR: IMPAIRED RESPONSIVENESS OF A MUTANT RECEPTOR IN FAMILIAL GLUCOCORTICOID DEFICIENCY

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The putative ACTH receptor gene has been identified on the basis of its tissue specific expression, structure, and limited expression data. We have expressed this gene in COS-7 cells and measured cAMP production in response to ACTH. An EC_{50} of 5.5 x 10^{-9} M for ACTH $_{(1-24)}$ was determined. The S74I mutant ACTH receptor gene that associates with the syndrome of familial glucocorticoid deficiency had an EC_{50} of 67 x 10^{-9} M. This discrepancy is consistent with the clinical data, and supports the hypothesis that this point mutation could account for the syndrome.

Identification of a cDNA clone as the ACTH receptor cDNA by Mountjoy et al [1] was based on detection of its expression in the adrenal cortex, its close homology to other members of the melanocortin family [2,3,4] and limited expression data in transfected Cloudman S91 melanoma cells which exhibited cAMP generation in response to a single dose of ACTH. More extensive expression studies have not been reported. Additional supportive evidence that this sequence encoded the ACTH receptor was provided by our demonstration that a homozygous point

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Abbreviations:
ACTH, Adrenocorticotropic Hormone; cDNA, complementary DNA; MSH, melanocyte stimulating hormone; cAMP, cyclic AMP; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; DOTAP, N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate.

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mutation in this gene tracked with the disease phenotype of familial glucocorticoid deficiency [5].

This syndrome, also known as hereditary unresponsiveness to ACTH, was first described by Shepherd *et al*, [6] and is characterized by hypoglycaemic episodes, convulsions, progressive hyperpigmentation, weakness, failure to thrive and excessively frequent and severe infections in early childhood. Amongst the 46 published cases there are 18 actual or presumed deaths from this syndrome within the first two years of life. Biochemical findings in untreated patients include elevated endogenous plasma ACTH levels and low or even undetectable basal levels of serum cortisol, which are subnormally responsive to exogenous $ACTH_{(1-24)}$ (Synacthen). Plasma renin and aldosterone levels are normal, in striking contrast to classical Addison's disease. Pathological studies of the adrenal glands removed post mortem from patients with familial glucocorticoid deficiency demonstrate atrophy of zona fasciculata and reticularis, with preservation of the zona glomerulosa and a normal medulla [6 - 8]. These findings have led to proposals that developmental defects of the adrenals, abnormalities of the ACTH receptor, or intracellular signalling defects downstream to the receptor are responsible for this disease. Our report of a point mutation that converts $Serine_{74}$ of the ACTH receptor to Isoleucine is currently the only such report of a mutation at this locus.

In the present study the function of the normal and mutant gene product was assessed by expression in eukaryotic cells, using cAMP release after stimulation with ACTH as an index of activity. Our results support the proposal that this clone is the ACTH receptor and that the S74I mutation is responsible for the disease in the family investigated.

PATIENTS, MATERIALS AND METHODS

ACTH receptor expression vector construction: Leucocyte genomic DNA from a normal individual and the proband from the family affected with familial glucocorticoid deficiency was subjected to amplification of the ACTH receptor gene using PCR and the oligonucleotide primers 656S and 1617A as described previously [5]. PCR products were subcloned into the vector pCRII (Invitrogen, San Diego, USA). The *EcoR1* fragment containing the entire subcloned PCR product was excised and inserted into the expression vector pcDNA1 (Invitrogen). Both normal and mutant ACTH receptors were sequenced in full in order to exclude the possibility of PCR generated mutations.

Cell lines and transfection procedure: COS-7 cells (CRL 1651, ATCC, Rockville, USA) were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, USA), supplemented with 10% fetal bovine serum, penicillin (50

U/ml), streptomycin (50 μg/ml) and L-glutamine (1 mM) at 37°C in a 5% CO_2 atmosphere. The cells were plated on 30-mm tissue culture dishes (5 x 10^5cells/plate) and grown overnight to about 80% confluence. After changing the medium the culture dishes were incubated for 6 h with 1 ml/plate transfection reagent/DNA mixture containing 5 μg of the expression vector encoding either the normal or S74I mutant human ACTH receptor, 12 μg of DOTAP (Boehringer Mannheim Biochemica, Mannheim, Germany) and Hepes buffered saline (Hepes 20 mmol/l, NaCl 150 mml/l, pH 7.4). The cells were co-transfected with 0.5 μg of pCH110, a β-galactosidase expression vector. An equal number of control cells were transfected with pCH110 alone. After replacing the transfection mixture with 2ml of supplemented medium the cells were incubated as described above.

ACTH stimulation and cAMP assay: 48 h after transfection the cells were washed twice with 1 ml DMEM. In the presence of 1mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, USA) in 0.5 ml DMEM per well, cells were stimulated with ACTH $_{(1-24)}$ (Sigma, St. Louis, USA) at various concentrations. After 60 min incubation medium was harvested and boiled for 3 min. cAMP was determined by a protein binding method [9]. Results reported for each transfected cell type represent cAMP values from 6 culture dishes for each ACTH concentration, and are represented as percent change to allow comparison between experiments.

β-galactosidase assay: Cells were washed twice with phosphate buffered saline (pH 7.4) before disruption by three cycles of freeze-thaw. β-galactosidase activity was measured in the cell lysate by a colorimetric enzyme assay method [10]. Statistical evaluation was performed using the single-factor Anova test using Microsoft Excel version 4.0.

RESULTS

The normal and S74I mutant ACTH receptor expression vectors were each co-transfected into COS-7 cells with the β-galactosidase expression vector and cells stimulated as described. Extracellular cAMP results were related to β-galactosidase activity which did not differ significantly between the cells transfected with the normal or mutant ACTH receptor or with pCH110 alone. Figure 1 shows the percentage increase of cAMP that accumulated in the extracellular medium over a period of 60 min after stimulation with ACTH $_{(1-24)}$ at final concentrations ranging from 10^{-10} to 3 x 10^{-7} M. In the unstimulated state there was a suggestion of constitutive activity in the normal and mutant receptors which was suppressed at low concentrations of ACTH. For this reason we used the mean cAMP levels after stimulation with 10^{-10} M ACTH as a reference point (100%). At this concentration of ACTH, absolute concentrations of cAMP did not differ significantly among the three groups within a single experiment.

Cells transfected with the normal ACTH receptor responded with a 5- to 6-fold increase in extracellular cAMP between ACTH concentrations of 10^{-9} and 10^{-8} M. Untransfected COS-7 cells also produced a significant cAMP response, indicating the existence of an endogenous ACTH or MSH receptor. However the differences

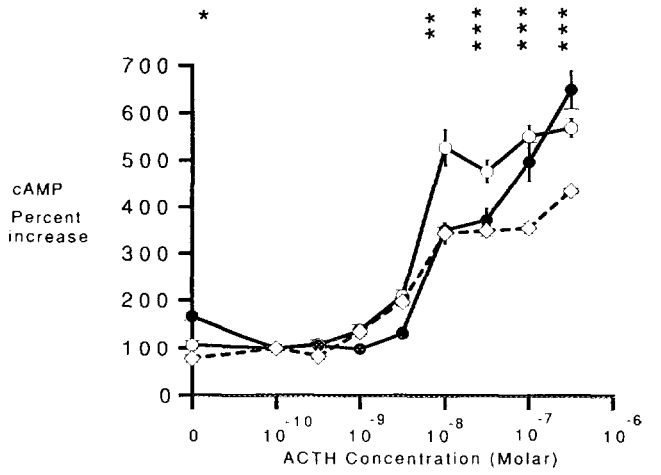

Fig. 1. Extracellular cAMP accumulation in medium from COS-7 cells transfected with the normal (open circles) and S74I mutant (closed circles) ACTH receptor in response to increasing concentrations of $ACTH_{(1-24)}$. The broken line (open diamonds) demonstrates a background cAMP signal from COS-7 cells transfected only with the β-galactosidase expression vector. Symbols represent the means (± SEM) of six transfections. Statistical significance of the difference between the normal receptor and background (endogenous) cAMP signal is represented by the asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

between the endogenous and normal ACTH receptors was highly significant above 3×10^{-9} M as indicated in Figure 1. These differences are illustrated more clearly when the background (endogenous) cAMP signal is subtracted from cAMP levels

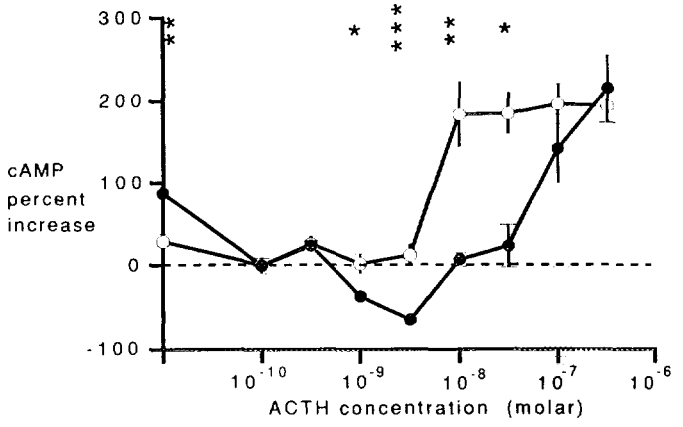

Fig. 2. Extracellular cAMP accumulation in medium from COS-7 cells transfected with the normal (open circles) and S74I mutant (closed circles) ACTH receptor after subtraction of the background (endogenous) cAMP signal of untransfected cells. Symbols represent the means (± SEM) of six transfections. Statistical significance of the difference between the normal and mutant receptors cAMP signal is represented by the asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

obtained from the cells expressing the normal or mutant ACTH receptors. Figure 2 shows the percentage increase of cAMP of both groups after this subtraction, and demonstrates very clearly the highly significant difference of the cAMP response between the normal and mutant ACTH receptors at ACTH concentrations ranging from 10^{-9} to 3 x 10^{-8} M. At ACTH concentrations above 3 x 10^{-8} M an increase of cAMP in cells transfected with the mutant ACTH receptor is observed and this response reaches a maximum at 3 x 10^{-7} M, suggesting a shift of the dose response curve to the right. Calculated EC_{50} values are 5.5 x 10^{-9} M (normal receptor) and 67 x 10^{-9} M (S74I mutant receptor) indicating a 12-fold shift to the right of this index.

DISCUSSION

The cloning of a family of genes encoding the melanocortin receptors was reported in 1992 [1]. Identification of one of these gene products as the human ACTH receptor was based on studies of the mRNA tissue distribution demonstrating its exclusive expression in the adrenal, and limited evidence of cAMP generation in Cloudman S91 melanoma cells. Using transient expression in COS-7 cells we have been able to confirm the ACTH responsiveness conferred by transfection of this gene which lends support to its identification as an ACTH receptor. These studies suggest an EC_{50} for $ACTH_{(1-24)}$ with the wild type receptor of 5.5 x 10^{-9} M, a figure that is remarkably similar to that of 5 x 10^{-9} M found for the ACTH response in bovine adrenal cells [11]. Thus the functional studies with the wild type receptor are compatible with the proposal that this is indeed the ACTH receptor. Steroid production by adrenal cells occurs at significantly lower ACTH concentrations [11], and recent work suggests that signalling via T-type calcium channels may be involved at lower ACTH concentrations [12].

Further support for the identification of this gene as being the ACTH receptor comes from our studies with patients whose clinical condition has been postulated as resulting from a defect of the ACTH receptor. Thus as previously reported we identified a homozygous mutation in two affected siblings. This mutation results in the substitution of $serine_{74}$ by an isoleucine residue and co-segregates with the disease [5]. In this report we identify its impaired function which results in a shift of the dose response curve to the right predicting an EC_{50} of 67 x 10^{-9} M.

These findings would predict that if the patient were able to increase ACTH levels approximately 12-fold, then the receptor would transmit an equivalent signal to

that produced by the normal receptor. This is precisely what happens in that ACTH levels are in the range of 300 - 1000 ng/l, approximately 12 times normal. The elevated levels of ACTH will have an unopposed action on cutaneous MSH receptors (which have similar affinities for γ-MSH and ACTH), and this results in the deep pigmentation that is characteristic of this syndrome.

The role of serine 74 is not known, although this residue is conserved amongst all members of the melanocortin receptor family. The free hydroxyl residue would be predicted to be directed towards the ligand binding pocket and away from the membrane in the amphipathic α-helix that makes up the second transmembrane domain of this receptor. Therefore it is probable that it is involved in a hydrogen bonding interaction either with the MSH core sequence of the ligand, or with another region of the receptor. This function could not be undertaken by the hydrophobic side chain of isoleucine. Undoubtedly a greater understanding of the functional role of this and other residues in the ACTH receptor will come in the future from site-directed mutagenesis studies.

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