INHIBITION OF ACTH-STIMULATED STEROIDOGENESIS IN ISOLATED RAT ADRENAL CELLS TREATED WITH NEURAMINIDASE

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SUMMARY

The possible role of membrane sialic acid in the action of ACTH was investigated in rat adrenal cells. After treatment with neuraminidase, the cells showed a diminished steroidogenic response to ACTH while the response to cyclic AMP and dibutyryl cyclic AMP was unaffected. 11β-hydroxylation of deoxycorticosterone (DOC) was also not impaired. Dose response curves for three ACTH peptides ($ACTH_{1-39}$, $ACTH_{1-24}$ and $ACTH_{1-10}$) with neuraminidase treated cells suggest that sialic acid residues on the glycoproteins of the plasma membrane may either impart affinity to the plasma membrane for ACTH molecule or facilitate transmission of the signal arising from ACTH-receptor interaction to the catalytic site of adenyl cyclase.

INTRODUCTION

The first step in the action of ACTH on adrenocortical cells appears to be its interaction with the plasma membrane (1,2). Although a considerable amount of work has been done on the structure - activity relationship in the ACTH molecule, virtually nothing is known about the components of the adrenocortical cell membrane that may be involved in the interaction with ACTH. It is generally accepted that there are distinct "binding" and "receptor-activating" sites on the ACTH molecule; the former is believed to consist of the basic amino acids at position 15-18 and the latter resides in the N-terminal decapeptide of the naturally occurring 39 amino acid peptide (3-6). Since the ACTH molecule contains basic amino acids in the binding site as well as in the receptor-activating site (histidine and arginine at positions 6 and 8 respectively) we reasoned that some negatively charged group on the plasma membrane of adrenocortical cells may be involved in the interaction with one or both of the sites on the ACTH

molecule. Sialic acid is known to be a component of membrane glycoproteins in several types of mammalian cells and is also known to contribute the negative charge to the cell surface (7). The function of sialic acid in a variety of glycoproteins and cell surfaces has been studied by observing the effects of selectively removing this residue by digestion with neuraminidase (8,9). Therefore, to test the possibility of sialic acid being involved in the interaction of ACTH with the plasma membrane of adrenocortical cells, we have treated these cells with neuraminidase and then tested their response to ACTH in terms of corticosterone production.

MATERIALS

Trypsin (TRSF-IGA 150 U/mg) and lima bean trypsin inhibitor (LBI) were purchased from Worthington Biochemical Corporation; collagenase (Serva, 387 Mandl U/mg) from Gallard Schlesinger Chemical Manufacturing Corp.; Pentex Bovine Serum albumin, fraction V powder, fatty acid poor, from Miles Laboratories; Neuraminidase of Cl. perfringens, type VI chromatographically purified (0.78 U/mg protein) cyclic AMP (CAMP) and dibutyryl cyclic AMP (DBCAMP) from Sigma Chemical Co.; and ACTH, U.S.P. corticotropin reference standard, from U.S. Pharmacopeial Convention, Inc. Eight hundred μUnits (sub cutaneous) of this preparation are equivalent to 1 μMole of the purified αs-ACTH obtained from Dr. C. H. Li.

METHODS

Rat adrenal cell suspensions were prepared in Krebs Ringer bicarbonate, pH 7.4, containing 0.2% glucose (KRBG) as described before (10,11) with a minor modification. Instead of using glass marbles and shaking in a Dubnoff metabolic incubator, mechanical agitation during collagenase-trypsin treatment was achieved with a magnetic stirring bar (60-70 r.p.m.).

The cells were suspended at a concentration of about 10^6 cells/ml of .01M phosphate buffer, pH 6.5, containing 0.85% NaCl, 0.2% glucose, 0.5% BSA and 0.1% trypsin inhibitor. Aliquots of the cell suspension were incubated at

37° for 15 minutes, neuraminidase was added and the incubation continued for the times designated in the tables. The suspension was next diluted with an equal volume of the same buffer and centrifuged for 20 minutes, final speed (100xg) being achieved slowly in the first 10 minutes. The sedimented cells were washed with KRBG, pH 7.4, (5 X original incubation volume) and the centrifugation repeated as above. Finally the cells were suspended in an appropriate volume of KRBG containing .75% BSA and 0.15% trypsin inhibitor to give a concentration of 1-2 x 10^5 cells/ml.

In the final incubation each beaker contained 1 ml of cell suspension, test substances dissolved in KRBG and an appropriate volume of KRBG to give a total volume of 1.5 ml. Incubations were carried out in duplicate for 2 hrs. at 37° in a Dubnoff metabolic incubator (64 shakes/minute) under an atmosphere of 5% CO_2:95% O_2. Corticosterone was measured fluorometrically by the method of Silber et al (12) and averaged values of duplicates are reported as nMoles/ 10^5 cells/2 hrs.

Sialic acid was measured according to Warren (13) in the supernatant obtained by centrifugation of the cell suspension after neuraminidase treatment. In the experiments where sialic acid was measured glucose was omitted from all solutions that came in contact with the cells up to and including the neuraminidase step because it interferes with the assay.

RESULTS AND DISCUSSION

Maximum stimulation of steroidogenesis in our cell suspensions is observed with 0.5-1 nM ACTH, 7-10 mM CAMP or about 0.5 mM DBCAMP. When maximally stimulated with any of these agents the rate of corticosterone production, which after a lag period of about 15 mins. is linear for at least up to 2 hrs., varies from 2-4 nMoles/10^5 cells/2 hrs. in different experiments. In the absence of these stimulating agents the cells do not synthesize any detectable amount of corticosterone (< .05 nMoles/10^5 cells) from endogenous precursors.

Table 1 shows the effect of treatment with increasing concentrations of neuraminidase on the subsequent response of the cells to different concentrations

Table 1. Effect of Neuraminidase Treatment on the Response of Adrenal Cells to ACTH.

Exp.	ACTH added nM	Neuraminidase mU/ml						
		0	2	5	10	20	25	50
		Corticosterone formed, nMoles/10^5 cells/2 hrs.						
1.	.25	3.9	3.7	3.3	3.1	3.1		
	1.0	4.3	4.3	4.0	4.0	3.7		
2.	.25	2.6			1.2		1.3	1.4
	.5	3.6			2.6		2.3	2.1
	1.0	3.8			3.0		2.8	2.8

Cells were suspended in .01M phosphate buffer, pH 6.5, containing 0.85% NaCl, 0.5% BSA and 0.1% trypsin inhibitor. After a 15' preincubation, neuraminidase was added and the incubation continued for 1 hour more. The cells were sedimented by centrifugation at 100xg, washed with KRBG (5 times the original incubation volume), suspended in KRBG containing BSA and trypsin inhibitor and incubated with ACTH for 2 hours as described in the "Methods".

of ACTH. After treatment with neuraminidase the response of cells to ACTH was substantially inhibited; greater inhibition being observed at lower ACTH concentrations. The maximum effect of neuraminidase was observed around 10-20 mU/ml. At these concentrations of the enzyme no lysis of cells was observed although concentrations greater than 50 mU/ml produced lysis in a fair percentage of cells. Therefore, in subsequent experiments 20 mU/ml concentration of the enzyme was routinely used.

Table 2 shows that 15 minute contact of the cells with the enzyme at a concentration of 20 mU/ml was sufficient to produce maximum effect on the subsequent response of the cells to ACTH.

When the sialic acid was measured in the medium after neuraminidase treatment for different times it was found that maximum release of the sialic acid occurred within 30 minutes. In three different experiments with 20 mU/ml neuraminidase, the amount of sialic acid released in 30 minutes was 9.5, 10.1 and 14.8 nMoles/

Table 2. Effect of Time of Neuraminidase Treatment.

Time of Neuraminidase Treatment	ACTH added nM	Control Cells	Neuraminidase treated Cells
		Corticosterone formed, nMoles/10^5 cells/2 hrs.	
15'	.06	0.9	0.4
	.12	1.5	0.8
30'	.06	0.9	0.3
	.12	1.4	0.9
60'	.06	0.8	0.4
	.12	1.5	0.9

Neuraminidase concentration was 20 mU/ml. Other experimental conditions same as in Table 1.

10^6 cells respectively. Control cells that were incubated without neuraminidase did not show measurable release of sialic acid into the medium. (< 2 nMoles/10^6 cells).

Since neuraminidase treatment resulted in cells that showed a diminished steroidogenic response to ACTH, it was necessary to check the response of such cells to CAMP. The data reported in table 3 shows that despite the alteration in response to ACTH, the neuraminidase-treated cells responded normally to different doses of CAMP and DBCAMP. These results showed that the reduced steroidogenic response of adrenal cells after neuraminidase treatment must have been due to an impairment of some function prior to, rather than after the formation of cyclic AMP. This conclusion is also supported by the fact that pyruvate-supported 11β-hydroxylation of DOC (11) in the neuraminidase treated cells was the same as in control cells.

If sialic acid was involved in a specific interaction with the "binding site" on the ACTH molecule (basic amino acids at positions 15-18) then the response of neuraminidase treated cells to the N-terminal decapeptide ($ACTH_{1-10}$) should not be altered. This peptide has been shown to stimulate corticosterone

Table 3. Effect of Neuraminidase Treatment on Some Intracellular Functions of the Adrenal Cells.

Additions	Control Cells	Neuraminidase-Treated Cells
	Corticosterone formed, nMoles/10^5 cells/2 hrs.	
ACTH .1 nM	1.0	0.5
" .2 "	1.7	1.1
CAMP 2.5 mM	1.2	1.2
" 7.5 "	2.4	2.5
DBCAMP 25 µM	0.6	0.7
" 50 "	1.4	1.2
" 100 "	1.6	1.9
" 500 "	2.5	2.4
DOC 180 nMoles	15.6	14.7
" " + Pyruvate 2mM	24.9	23.6

Neuraminidase 20 mU/ml; Time of treatment - 30 mins.

production in the isolated adrenal cells by Schwyzer et al. although much higher concentrations are required to show the effect (5). In Fig. 1 are shown the log dose response curves for $ACTH_{1-39}$, $ACTH_{1-24}$ and $ACTH_{1-10}$ with control and neuraminidase treated cells and it is clear that the latter show a decreased response to all three peptides. After neuraminidase treatment of the cells, there was 10-15% reduction in the B_{max} (maximum rate of corticosterone production by control cells); however, the concentration of hormones required to produce 1/2 B_{max} (A_{50}) was found to be increased by 50-100% in different experiments. (See Sayers et al. (14) for a discussion of the terms B_{max} and A_{50} for ACTH and isolated adrenal cells.)

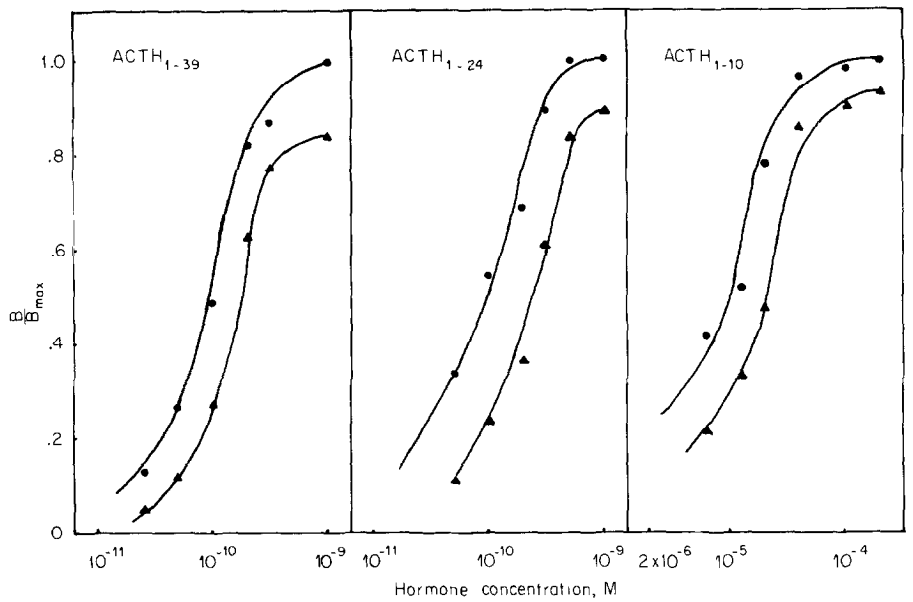

Fig. 1 Log Dose Response Curves fo $ACTH_{1-39}$, $ACTH_{1-24}$ and $ACTH_{1-10}$ with Control (• ——— •) and neuraminidase treated cells (▲——▲). The points are the observed values of B/B_{max} where B is the rate of corticosterone production and B_{max} is the rate of corticosterone production by control cells with maximally stimulating levels of the hormone.

Our data suggest at least two possibilities (i) Sialic acid on the adrenal cortex cell membrane may provide affinity for the ACTH molecule and thereby facilitate activation of the receptor. Although the N-terminal decapeptide has been shown to contain the "receptor-activating" site the possibility that some amino acid(s) in this region contribute to the "affinity" for the receptor can not be ruled out. (ii) Sialic acid may facilitate "transmission" of the signal arising from ACTH-receptor interaction to adenyl cyclase. Such a role for membrane sialic acid in the action of insulin on fat cells has been suggested by Cuatrecasas (15). Studies concerning cyclic AMP production in and the binding of ACTH to the neuraminidase treated cells are currently in progress.

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