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# THE EFFECT OF CELL MEMBRANE ALTERATION ON GLUCOCORTICOID UPTAKE BY THE AtT-20/D-1 TARGET CELL

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## Summary

The glucocorticoid-sensitive AtT-20/D-1 cell line was used to study cellular uptake of glucocorticoids. A previous observation that glucocorticoid uptake by these cells was temperature dependent had prompted us to postulate that glucocorticoids entered the cell by a temperature-sensitive transport process located in the cell membrane. Attempts were then made to perturb the membrane mechanism. In some of these experiments, intact cells were treated with neuraminidase or pronase. The release of sialic acid in the case of neuraminidase treatment and of sialic acid and cell surface peptides in the case of pronase treatment demonstrated that the enzymes were effective. Approx. 60% of total cellular sialic acid was released by a 15 min incubation with 20 μg/ml neuraminidase at 25°C. The treated cells appeared to be viable, in that they continued to produce corticotropin at a normal rate, yet intact cell glucocorticoid binding at both 4 and 25°C was only 20—30% of that of untreated cells. Treatment with pronase also caused steroid uptake at 4 and 25°C to be reduced, although the extent of reduction was less than that seen following neuraminidase treatment.

In other experiments, the effect of exposure of AtT-20/D-1 cells to ethanol or dimethyl sulfoxide was determined. The solvent concentrations used (0.5—10%) did not alter cell viability significantly, and the ability of the cytosol receptor to bind steroid in a cell-free preparation was unimpaired. However, incubation of intact cells with 10% (v/v) dimethyl sulfoxide or ethanol resulted in an 80—90% decrease in steroid uptake at 25°C.

We conclude that steroid uptake by the intact cell can be perturbed by treatments which do not affect the cytosol receptor or alter cell viability. These

* Trivial names used are: triamcinolone acetonide, 9α-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione-16α,17α-acetonide; dexamethasone, 9α-fluoro-11β,17,21-trihydroxy-16α-methyl-pregna-1,4-diene-3,20-dione.

results support the postulate that glucocorticoids enter the AtT-20/D-1 cell by a specific membrane-associated mechanism.

## Introduction

It has been previously assumed that steroids freely diffuse into all cells and that the interaction of a steroid hormone with its cytosol receptor is the first definitive step in the mechanism of steroid hormone action. Our studies of glucocorticoid uptake by the mouse pituitary tumor cell line AtT-20/D-1 have produced results which are not explained by this simple model. The uptake process by these cells has temperature-dependent characteristics which suggest that, during steroid uptake, a cell membrane-mediated step occurs prior to formation of the glucocorticoid-receptor complex [1]. We have studied the effects of various enzymes and other agents on uptake since exposure of intact cells to the actions of hydrolytic enzymes has been frequently used by others to probe mechanisms located in the cell membrane [2—4]. Steroid uptake by the AtT-20 cell is sensitive to the effects of hydrolytic enzyme action [5].

In this paper the effects on steroid uptake of two enzymes, neuraminidase and pronase, and two solvents, ethanol and dimethyl sulfoxide, are reported. These results support the concept that steroid uptake involves a specific membrane-associated mechanism.

## Materials and Methods

Neuraminidase (EC 3.2.1.18) was obtained from Worthington Chemical Co. and pronase (EC 3.4.21.4 and 3.4.24.4) from Calbiochem. [1,2,4-$^3$H]-triamcinolone acetonide (4 Ci/mM) was obtained from Schwartz-Mann. Unless otherwise noted, all other chemicals were obtained from Sigma Chemical Co.

*Cell culture.* AtT-20/D-1 (American Type Culture Collection CCl-89) cells were grown in suspension culture in 9-l spinner flasks containing Eagle's minimal essential medium (Dulbecco's modification) (North American Biologicals) supplemented with a 15% horse serum and a 2.5% fetal calf serum (Grand Island Biologicals), under a 10% $CO_2$ atmosphere. The cells were allowed to settle briefly, and the pellet was harvested by gentle aspiration through a large-bore pipette.

*Steroid uptake.* Cells were diluted with fresh medium to a final concentration of $5 \cdot 10^6$/ml. Incubations were begun with the addition of labeled triamcinolone acetonide dissolved in ethanol to a concentration of $1.15 \cdot 10^{-8}$ M. A 500-fold excess of unlabeled dexamethasone, also dissolved in ethanol, was added to duplicate samples to determine displacable or "specific" binding as previously described by us [1]. Displacable or "specific" binding was greater than 80% of total binding in most experiments. The final concentration of ethanol in the incubation medium was less than 0.1%. Incubations were terminated by adding 1-ml aliquots of the incubation mixtures to tubes containing 4 ml of ice-cold Tris/saline buffer (10 mM Tris · HCl, 154 mM NaCl, pH 7.5). The tubes were centrifuged for 5 min at 4°C at 800 × *g*. The cell pellets were washed three times by resuspending them with vigorous vortexing

in 2 ml of ice-cold Tris/saline buffer and centrifuging them as before. The radioactivity retained in the pellet was determined by direct extraction of the non-covalently bound steroid into counting fluid as previously described [1].

*Enzyme incubations.* Enzyme incubations were performed in Eagle's medium containing 10 mM Tricine, pH 7.5, and no serum. Following enzyme addition the mixture was incubated for 15 min at 25°C. The enzyme incubation was stopped by rapid cooling, and the cells were washed three times in Tris/saline buffer containing 10% horse serum. The washed cells were resuspended in fresh medium for determination of steroid uptake.

*Ethanol and dimethyl sulfoxide treatment.* Cells were suspended in medium without serum and incubated at 25°C with various concentrations of ethanol or dimethyl sulfoxide for 2 h. The ability of these treated cells to take up labeled triamcinoline acetonide was then determined, without changing the medium.

*Miscellaneous assays.* Protein concentration was determined, using bovine serum albumin as standard [6]. Corticotropin was determined by radioimmunoassay [7]. Sialic acid was determined by the thiobarbituric acid method with *N*-acetylneuraminic acid as standard [8].

*Lactoperoxidase iodination.* This procedure, based on methods described by Morrison [9] and others [10,11] was performed in a total volume of 1 ml of phosphate-buffered saline containing $3 \cdot 10^8$ cells. The concentrations of reactants were: 0.43 $\mu$M lactoperoxidase, 1 $\mu$M KI and 100 $\mu$Ci $Na^{125}I$/ml of reaction mixture. 2.3 mM $H_2O_2$ was added in 20-$\mu$l aliquots at 15-s intervals. 2 ml of cold Tris/buffered saline containing 1 mM KI and 10% horse serum were then added, and the mixture was centrifuged at 800 × *g* for 5 min. The cell pellet was washed three more times in the same solution by alternate suspension and centrifugation. The concentrations of lactoperoxidase and $H_2O_2$ were determined by their absorbance, using published extinction coefficients [10].

## Results

*Effect of neuraminidase on steroid uptake*

Steroid uptake by AtT-20 cells can be decreased by exposing them to neuraminidase [5]. The results shown in Fig. 1 demonstrate that as little as 5 $\mu$g/ml of neuraminidase inhibited intact cell uptake at 25°C by 50%. Prior treatment with neuraminidase also markedly diminished the small amount of intact cell binding normally seen at 4°C. Neuraminidase which had been inactivated by heating at 80°C for 15 min did not significantly affect uptake at either 25 or 4°C.

The ability of neuraminidase to remove sialic acid groups from intact AtT-20 cells was determined by measuring the total sialic acid remaining in the cell pellet following enzyme treatment. The results of treatment with various concentrations of the enzyme are shown in Fig. 2. The decrease in total cellular sialic acid was concentration dependent, the maximal effect being observed in this experiment at an enzyme concentration of 10—20 $\mu$g/ml although in other experiments sialic acid content was reduced even further at concentrations of 20 $\mu$g/ml or greater. Fig. 2 also illustrates that the 15 min enzyme incubation

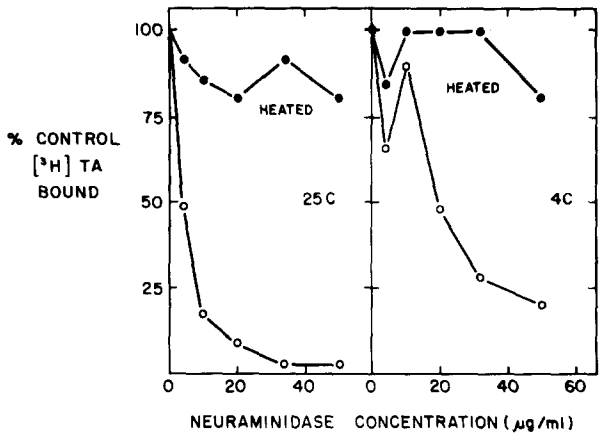


Fig. 1. The effect of neuraminidase on cell uptake at 25 and 4°C. Enzyme incubations were performed as described in Materials and Methods using neuraminidase concentrations shown on the abscissa. Steroid uptake was determined after a 10 min incubation at either 25 or 4°C. Heated neuraminidase was prepared by heating the enzyme, dissolved in Eagle's medium at 80°C for 15 min. TA, triamcinolone acetonide.

routinely used was adequate for the neuraminidase to have a near-maximal effect. The correlation between sialic acid release caused by incubation with various concentrations of neuraminidase and the extent to which steroid uptake was inhibited by these treatments was excellent ($r = 0.96$). On the other hand, maximally effective concentrations of pronase only resulted in a 24% fall in cell sialic acid and correlation between sialic acid reduction and inhibition of steroid uptake was minimal ($r = 0.60$).

The extent to which neuraminidase caused the release of cell surface peptides was determined by labeling the cell surface with [$^{125}$I] iodine using a lactoperoxidase-catalyzed reaction. Cells thus labeled were then treated with neuraminidase. Less than 10% of the iodopeptides were released by

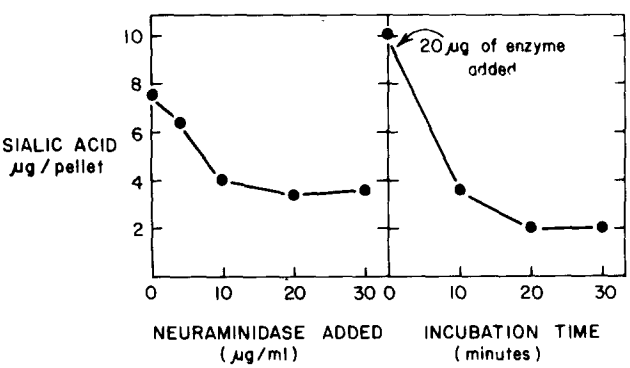


Fig. 2. Release of cell sialic acid by neuraminidase treatment. Aliquots of cells ($10^7$) were treated with various concentrations of neuraminidase for 15 min (left) or with 20 μg/ml neuraminidase at 25°C for various time intervals (right). Following incubation the cells were cooled to 4°C and washed three times with ice-cold Tris/saline. The sialic acid present in the cell pellets was extracted by heating at 90°C for 15 min in 0.05 M $H_2SO_4$. The content of sialic acid was determined in this extract by the thiobarbituric acid procedure (see Materials and Methods).

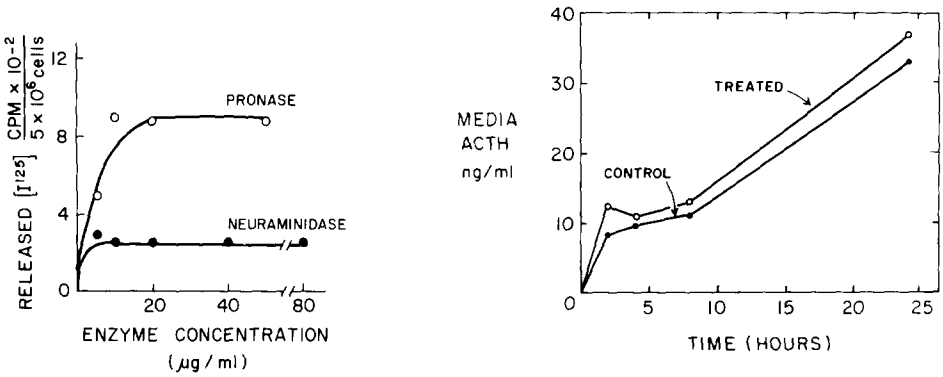


Fig. 3. Release of cell protein by treatment with pronase or neuraminidase. Cells were treated with various concentrations of enzyme at 25°C for 15 min. One group of cells had been previously iodinated with $^{125}I$ using the lactoperoxidase technique (see Materials and Methods). A total of 2330 cpm $^{125}I$ were incorporated into the aliquots of cells used for each determination. Following enzyme treatment, these cells were centrifuged, and the supernatants were counted in a $\gamma$-scintillation spectrometer to determine the amount of $^{125}I$-labeled peptides or proteins released from the cells. The radioactivity of supernatants of cells treated similarly in the absence of enzyme was used to correct for non-specific loss of [$^{125}I$]-iodine. This was approx. 10% of the total radioactivity released.

Fig. 4. Effect of neuraminidase treatment on corticotropin production by intact cells. Cells were treated with 20 μg/ml neuraminidase at 25°C for 15 min. The control group was not exposed to the enzyme. Both groups of cells were then incubated in Falcon Flasks at a starting concentration of $2 \cdot 10^5$/ml, and the concentration of radioimmunoreactive corticotropin released into the culture medium was determined at various times thereafter. The medium was withdrawn and frozen for subsequent corticotropin radioimmunoassay.

neuraminidase (Fig. 4). This amount was small in comparison to that released by pronase under similar conditions.

The possibility that neuraminidase treatment prevented cellular steroid uptake by causing non-specific damage to the cell was examined. It has been previously shown that cytosol receptor levels are not affected [5]. Protein biosynthesis was used as another index of cell viability and functional integrity since it was thought possible that cell membrane alteration might lead to spurious estimates of viability by the dye-exclusion method. AtT-20 cells were incubated with a sterile 20 μg/ml solution of neuraminidase for 15 min which reduced their ability to take up labeled steroid at 25°C to 50% of control (not shown). The cells were washed and suspended in fresh medium. The rate of cellular protein synthesis was determined by observing the incorporation of radioactivity from a pulse of [$^{14}C$]valine into the trichloroacetic acid-precipitable cell fraction. Protein synthesis appeared to continue unabated after neuraminidase treatment (not shown). Corticotropin production is a characteristic differentiated function of these cells. Therefore, the ability of neuraminidase-treated cells to synthesize the polypeptide hormone, corticotropin was also determined (Fig. 4) in order to verify cell viability by an independent method. The concentration of corticotropin present in the medium after 2, 4, 8 and 24 h of incubation at 37°C was determined. Corticotropin production was unaffected by neuraminidase treatment (Fig. 4) that reduced steroid binding by 50%. These results demonstrated that the neuraminidase-treated cells were viable and functional.

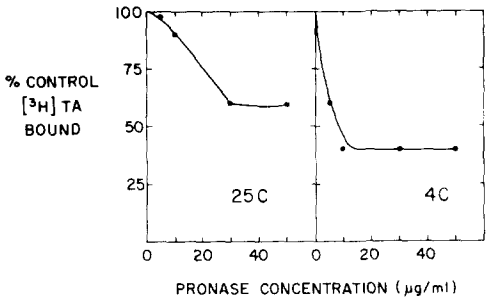


Fig. 5. The effect of pronase on [$^3$H]triamcinolone acetonide (TA) uptake at 25 and 4°C. Intact cells were treated with various concentrations of pronase at 25°C for 15 min (see Materials and Methods). The cells were washed, and the amount of [$^3$H]triamcinolone acetonide bound in 10 min was determined at 25 and 4°C.

*Effect of pronase on steroid uptake*

Maximally effective pronase concentrations were estimated by measuring the release of iodoproteins from cells iodinated using lactoperoxidase (see Materials and Methods). The reaction was completely dependent upon the presence of added $H_2O_2$ and lactoperoxidase and resulted in the iodination of seven molecular weight classes of proteins, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown). The results shown in Fig. 3 indicate that treatment with 10 μg/ml of pronase resulted in maximal release of iodopeptides from intact cells. The percentage of iodopeptides released was 43% and was similar to the amount released when iodinated HeLa cells were treated with pronase [12]. This concentration of pronase resulted in the loss of approx. 24% of cellular sialic acid (not shown) or approximately one-third of that released by neuraminidase.

The effect of pronase treatment on steroid uptake at 25 and 4° C is shown in Fig. 5. Uptake was only partially reduced by exposure to a pronase concentra-

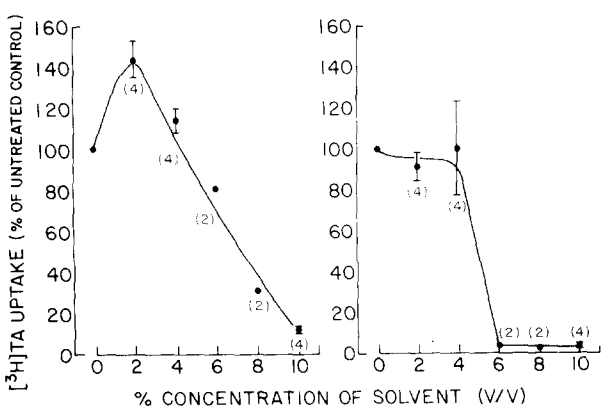


Fig. 6. The effects of dimethyl sulfoxide and ethanol on intact cell uptake. Intact cells were incubated with various concentrations of dimethyl sulfoxide (left) or ethanol (right) for 2 h, after which the amount of [$^3$H]triamcinolone acetonide (TA) bound in 10 min at 25°C was determined. Two separate experiments are combined. The numbers in parentheses under each point represent *n* and the bars indicate the standard error of the mean.

TABLE I

EFFECTS OF DIMETHYL SULFOXIDE AND ETHANOL ON CELL VIABILITY

Cells were suspended in Eagle's medium at a concentration of $5 \cdot 10^6$/ml. The solvents were added at the indicated concentrations and the cell/solvent mixture incubated at 25°C for 2 h. The cells, which normally grow as aggregates, were then trypsinized (0.25% trypsin-EDTA, 37°C for 10 min) and suspended in a solution of 0.05% nigrosin, 10% horse serum in phosphate-buffered saline, and counted. Cells which excluded the black nigrosin stain were considered viable.

| Solvent concentration (v/v) | Viability (%) |
|---|---|
| Untreated control | 81 |
| Ethanol | |
| 0.5 | 85 |
| 1.0 | 85 |
| 5.0 | 81 |
| 10.0 | 79 |
| Dimethyl sulfoxide | |
| 2.0 | 75 |
| 5.0 | 80 |
| 10.0 | 79 |

tion five times greater than that known to result in maximal proteolysis. Uptake at 4°C was slightly more sensitive than that at 25°C.

*Effect of ethanol and dimethyl sulfoxide on steroid uptake*

The results of our studies suggested that steroid uptake is affected by the functional state of the AtT-20/D-1 cell membrane. Ethanol and dimethyl sulfoxide, which are known to affect various cell membrane-associated functions, were examined for their effects on steroid uptake. The effects of several concentrations were tested from 0 to 10% (w/v). At low concentrations dimethyl sulfoxide had a stimulating effect on the rate of uptake (Fig. 6). Both ethanol and dimethyl sulfoxide caused a dramatic inhibition of uptake at higher concentrations (Fig. 6). There was little effect on cell viability as shown by the results in Table I. When either ethanol or dimethyl sulfoxide was used in maximally effective concentrations, cell viability, as determined by dye exclusion, was still 70% or greater.

Since the effectiveness of these solvents in inhibiting steroid uptake could not be ascribed to altered cell viability, their effects on binding capacity of the soluble cytosol receptor was examined. Cytosol was prepared from cells which had been treated with 10% ethanol or dimethyl sulfoxide. The ability of these cytosol preparations to bind [$^3$H]triamcinoline acetonide was then compared to that of control cytosol prepared from untreated cells. Neither ethanol nor dimethyl sulfoxide reduced cytosol binding.

## Discussion

The cells used in these experiments were originally derived from a radiation-induced mouse pituitary adenocarcinoma and produce corticotropin, the synthesis of which is inhibited by physiologic concentrations of glucocorticoids [16]. They have been previously shown to have a cytoplasmic receptor for

glucocorticoids which undergoes nuclear translocation [17]. The experiments reported in this paper show that intact AtT-20/D-1 cells treated with neuraminidase are less able to bind glucocorticoids than untreated or pronase-treated cells. This effect does not appear to depend upon cell damage, since specialized cell functions such as corticotropin synthesis proceed unchanged in neuraminidase-treated cells. It seemed possible that neuraminidase, or proteolytic contaminants of the neuraminidase preparation, caused this effect by releasing cell membrane proteins. However, the observation that, compared to pronase, neuraminidase caused no significant release of iodopeptides from lactoperoxidase-iodinated cells made that possibility unlikely. Furthermore, previous work has shown that neuraminidase treatment does not affect the binding of triamcinolone acetonide by the AtT-20 cytosol receptor [5]. There was an excellent correlation between the inhibitory effect of neuraminidase on steroid uptake and the release of cell sialic acid. Pronase reduced steroid uptake to a lesser extent, and this effect did not appear to correlate well with the release of sialic acid from the cells.

Ethanol and dimethyl sulfoxide also caused a reduction in steroid uptake. The data presented in this paper show that these effects were not due to cell death nor to a reduction in binding capacity of the intracellular receptor. The specific site of action of these solvents is uncertain since they are known to affect several cell functions, such as cell membrane transport of nucleosides [13], activity of some soluble enzymes [14] and the character of lysosomal membranes [15].

Although a precise explanation for these observations awaits a more complete understanding of glucocorticoid interactions with the intact cell, the experiments reported here demonstrate an effect on the ability of the intact cell to bind steroids. Since these results cannot be ascribed to changes in the soluble cytosol receptor they suggest that an intact and functional cell membrane is essential for normal glucocorticoid uptake by the target cell.

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