J. steroid Biochem. Vol. 23, No. 4, pp. 529–533, 1985
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0022-4731/85 $3.00 + 0.00
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SHORT COMMUNICATION

THE EFFECT OF MOLYBDATE ON GLUCOCORTICOID RECEPTOR TRANSFORMATION AND TRANSLOCATION IN INTACT, VIABLE AtT-20 MOUSE PITUITARY TUMOR CELLS

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(*Received* 4 *February* 1985)

Summary—The effect of 20 mM molybdate on the transformation and translocation of glucocorticoid receptors in intact AtT-20 mouse pituitary tumor cells was investigated.

To test whether transformation of the receptor was inhibited during *in vivo* incubations with both molybdate and glucocorticoid, the DEAE cellulose elution profile of extracted receptor was determined. It was found that receptors from both high speed cytosols and nuclear extracts were transformed.

To test whether translocation was affected by molybdate, the fraction of glucocorticoid–receptor complexes found in the nucleus was determined. At 37°C, in the absence of molybdate, 55–60%of the glucocorticoid receptor complexes were in the nuclear compartment. Molybdate did not effect the magnitude of nuclear translocation. Control studies suggested that the agent entered the cells, however. Cold exposure (0°C) reduced nuclear translocation to 20–25%.

It is concluded that *in vivo*, either molybdate is not able to interact with the receptor or transformation *in vivo* is not mediated by the same molybdate-sensitive mechanisms currently being studied using broken cell-preparations.

INTRODUCTION

We have been studying the interactions of glucocorticoids with the intact AtT-20 cell in order to elucidate which elements of the steroid-receptor–nuclear interaction are most important in determining biologic activity [1, 2]. In one approach to this problem we are trying to alter the magnitude of nuclear translocation and then determine how this change alters steroid biopotency. Several agents have been identified which decrease the magnitude of receptor transformation. Sodium molybdate is one such agent, but it has been investigated almost exclusively *in vitro*. If this agent is active in the intact cell, it could be helpful in quantitating the importance of nuclear binding on biologic activity. This paper reports the effect of sodium molybdate on *in vivo* transformation and translocation in the intact AtT-20 cell.

EXPERIMENTAL

Maintenance of cells and dilution of steroids have been described [3]. Cell viability was determined by the exclusion of Trypan blue.

Chemical agents

Stock solutions of sodium molybdate, at a concentration of 100 mM were prepared in media, adjusted to pH 7.5, filter sterilized, and stored at 4°C.

Measurement of nuclear translocation

AtT-20 cells, at a concentration of 5×10^6/ml, were incubated at 37°C in growth media either in the presence or absence of agent. After 1 h of preincubation, tritiated dexamethasone was added (final concentration 10 nM) and the cells incubated for an additional 3–4 h. Thereupon, cells were gathered by gentle centrifugation, (800 *g*, 5 min, 5°C), suspended in ice-cold Buffer I and duplicate aliquots removed for determination of intact cell binding. All subsequent steps were done in an ice bath. The washed pellet was resuspended in 2 ml of Buffer II and homogenized with 5 strokes of a metal dounce. Replicate aliquots of these homogenates were centrifuged at 800 *g*, 10 min, 5°C to prepare low-speed cytosol and crude nuclear pellets. Samples of cytosol were removed for radioactivity determinations. The pellets were washed once more with Buffer II before they, too were transferred to scintillation vials for radioactivity determination.

Additional flasks of cells were always included which were identical to those described except that they contained a large excess of nonradioactive dexamethasone (10 μM) so that nondisplaceable binding could be calculated.

Cell incubation for column chromatography

AtT-20 cells were suspended in growth media at a concentration of 10^7/ml. Where appropriate, they were made 20 mM in sodium molybdate and preincubated for 30 min at either 37 or 0°C. Thereupon, tritiated triamcinolone acetonide was added to achieve a final concentration of 10 nM. After an additional incubation period, all cells were placed in an ice bath, centrifuged, washed once with Buffer I and then homogenized in 2 ml of Buffer III using 5 strokes of a metal dounce. These homogenates were centrifuged for 10 min, 5°C, 800 *g* to prepare low-speed cytosols and crude nuclear pellets. If the cytosols were to be chromatographed they were further centrifuged at 100,000 *g* for 1 h at 4°C. If the nuclear receptors were to be analyzed, the pellets were washed with Buffer III to remove entrapped cytosol and then resuspended in 2 ml of Buffer IV for 30 min at 0°C to extract the receptor. These extracts were centrifuged for 5 min at 800 *g*, 5°C before chromatography.

Table 1. The effect of molybdate on nuclear translocation in the AtT-20 cell

		A	B	C
		37°C	0°C	Molybdate
1	Whole cell binding $\frac{\text{Experimental}}{\text{37°C Control}}$ (%)	100	46.0 ± 7.0	101 ± 12.9
2	$\frac{\text{Nuclear binding}}{\text{Whole cell binding}}$ (%)	54.9 ± 2.8	22.6 ± 2.2	61.5 ± 8.9
3	$\frac{\text{Nuclear binding}}{\text{Nuclear + cytosol binding}}$ (%)	61.4 ± 4.6	26.7 ± 6.3	62.9 ± 4.5
4	$\frac{\text{Nuclear + cytosol binding}}{\text{Whole cell}}$ (% recovery)	89.2 ± 2.3	103 ± 27	97.3 ± 9.1

AtT-20 cells were suspended in growth media at 5×10^6/ml. Six identical samples were prepared. Two were placed at 0°C while the others were maintained at 37°C. Two of the latter were supplemented with sufficient molybdate to make its final concentration 20 mM. All were incubated for 1 h to equilibrate. After that, one of each pair was made 10^{-5} M in nonradioactive dexamethasone and then all were made 10 nM in tritiated dexamethasone. After 3–4 h of incubation, two 1 ml aliquots of each were removed to determine intact cell displaceable binding. Another 5 ml aliquot was centrifuged, washed once with Buffer I and then dounced in 2 ml of Buffer III. These samples were centrifuged to prepare low-speed cytosol and crude nuclear pellets. Two-hundred μl aliquots of the cytosol were transferred to vials for radioactivity determination. The pellets were washed once in fresh Buffer III and then transferred to scintillation vials. Displaceable binding was calculated in each case. The amount of binding in each of the two subcellular fractions was related to the amount bound by the intact cell to determine % recovery. Whole cell binding values are expressed as a percentage of the value from the 37°C control. Values shown are mean ± SEM (n = 3).

Column chromatography

The nuclear extracts had to be chromatographed on Sephadex G-25 to remove KCl before application to the DEAE column. Specifically, Sephadex G-25 (Pharmacia, Piscataway, NJ) in Buffer V was packed in a 30 ml syringe which was 5.5 cm deep and 2.4 cm in dia. A 1 ml sample of extract was applied and thirty 2 ml fractions collected. All steps were done in a 5°C cold room. The radioactivity in 0.1 ml aliquots was determined. The material eluting in the void volume (generally tubes 5–9) was collected and applied to the DEAE column. DEAE chromatography has been described [3].

Other chemicals and assays

Protein was determined by the method of Lowry *et al.*[4] and DNA by the method of Burton[5]. The charcoal dextran procedure has been described [6]. Tritiated thymidine, 60 Ci/mmol, was from ICN (Irvine, CA).

Buffers

Buffer I: 10 mM Tris base + 154 mM NaCl, pH 7.5. Buffer II: 50 mM Tris base + 1 mM EDTA + 6 mM monothioglycerol, pH 7.5. Buffer III: 10 mM Tris base + 1 mM EDTA + 6 mM monthioglycerol + 20 mM sodium molybdate, pH 7.5. Buffer IV: Buffer III made 0.3 M KCl. Buffer V: 10 mM Tris base + 1 mM EDTA + 20 mM sodium molybdate, pH 7.5.

RESULTS

The effect of sodium molybdate and cold exposure on cell viability and growth were determined. Even at concentrations as high as 40 mM, molybdate did not diminish viability at 24 h (Trypan blue). On the other hand, after a comparable exposure to 0°C, all cells had died. The effect of molybdate on cell growth was evaluated during a 72 h exposure. At very low concentrations (1 mM) molybdate did not affect cell growth. However, at higher concentrations it caused a dose-dependent decrease in DNA accumulation compared to control (10% decrease at 10 mM, 60% decrease at 50 mM).

Table 1, column A, row 2, demonstrates that the fraction of steroid receptor complex translocated to the nucleus in control cells incubated at 37°C is 54.9 ± 2.8%. This result agrees with our previous finding [1] using a completely different technique of cell fractionation. If the cells were incubated at 0°C (column B) nuclear translocation was inhibited. Importantly, recovery is high in each case and approaches 100% (row 4).

To assess the effect of sodium molybdate (20 mM) the cells were first preincubated with the agent and then exposed to tritiated steroid (10 nM) for 3–4 h. At that point, cells were gathered and fractionated. The results are shown in Table 1 (column C). The first parameter to be considered is the amount of intact cell binding. Molybdate did not interfere with binding to the intact cell's receptor. Cold exposure, however, decreased binding. After fractionation, recovery of bound radioactivity was high in each case (Row 4). The results in Rows 2 and 3 demonstrate that molybdate did not alter nuclear translocation significantly but cold exposure did.

Although molybdate did not affect translocation, we wondered whether it had inhibited the structural changes that take place during receptor transformation [7]. To investigate this possibility, the effect of molybdate on the molecular form of the receptor incubated with steroid *in vivo* was analyzed by DEAE cellulose chromatography. Figure 1 (upper panel) demonstrates the results for the cytosolic receptor isolated from cells that had been incubated with tritiated triamcinolone acetonide in the presence of 20 mM molybdate at 37°C. The majority of the receptor eluted at the position of the transformed receptor [3].

Analysis of the nuclear form of the receptor was more difficult because the preparation had to be exposed to 0.3 M KCl to extract it from the nucleus. This extract, in turn, had to be chromatographed on Sephadex G-25 to remove KCl before DEAE chromatography. Both of these procedures (exposure to high salt and molecular sieve chromatography) are known to transform the receptor [7]. Thus, it was necessary to establish conditions under which untransformed receptor could be prepared for chromatography but still preserve its original form. It was found that this could

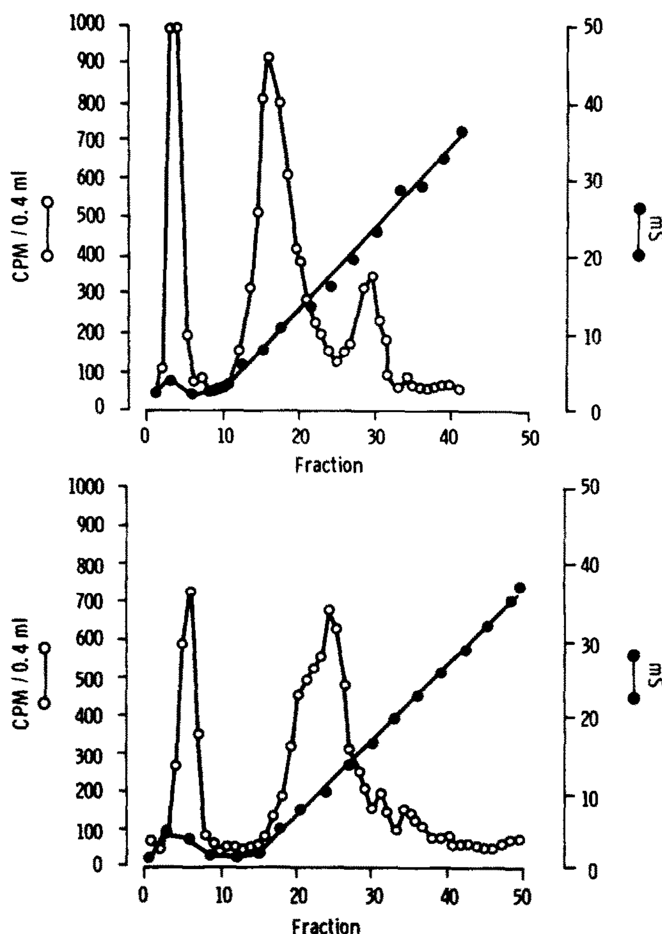

Fig. 1. DEAE chromatography of glucocorticoid receptor isolated from molybdate treated AtT-20 cells. Upper panel: AtT-20 cells were suspended in growth media at 37°C that was supplemented with 20 mM sodium molybdate and allowed to incubate for 30 min. Thereupon sufficient tritiated triamcinolone acetonide was added to make its final concentration 10 nM and the cells incubated for an additional 2 h at 37°C. The cells were pelleted, washed once with Buffer I and high-speed cytosol prepared in Buffer III. One ml was applied to the DEAE column and eluted. The ordinate on the right gives the conductivity of every third tube in mS (where S = siemen = 1 Ω^{-1}). Lower panel: The crude nuclear pellet prepared from the above cells was washed once with Buffer III and then extracted with Buffer IV for 30 min at 0°C. After centrifugation (10 min, 5°C, 800 *g*) 1 ml of the supernatant was applied to a Sephadex G-25 column equilibrated in Buffer V. The radioactivity which chromatographed in the exclusion volume was applied to the DEAE column.

be done if molybdate was included in all of the homogenization and preparative chromatography steps. The effectiveness of these inclusions is shown in Fig. 2.

Figure 1 (lower panel) gives the results of the nuclear receptor extracted from intact cells which had been incubated with tritiated triamcinolone acetonide at 37°C in the presence of 20 mM molybdate. As can be seen, this receptor eluted from DEAE at the position of the transformed species. Since the extraction procedure does not artefactually transform receptor (Fig. 2), it is concluded that the complex had been transformed in the intact cell even though 20 mM molybdate had been present throughout the steroid exposure.

These results suggest that molybdate does not prevent transformation of the receptor *in vivo*. Another possibility was that the molybdate did not enter the cell. To investigate this possibility two experiments were conducted. In the first, cells were grown in the presence or absence of 20 mM molybdate and their ability to incorporate tritiated thymidine measured. These results suggest that molybdate entered the cell, for within 6 h it caused a 50% reduction in their rate of incorporation of the thymidine (data not shown). In the second experiment, cytosol was prepared from cells that had been incubated *in vivo* with 20 mM molybdate and the ability of their glucocorticoid receptors to resist *in vitro* heat-transformation was studied. As a control, a sample was processed in a similar fashion except that its initial incubation was in molybdate-free media. As expected (Fig. 3 upper panel) receptor from control cell when heat transformed eluted at the position of the transformed receptor. On the other hand, a significant amount of the receptor from the molybdate-treated sample emerged at the position of the untransformed receptor (Fig. 3, lower panel). This indicates that sufficient molybdate was associated with the cells to inhibit *in vitro* transformation. Other studies (not shown) revealed that additional washing of the

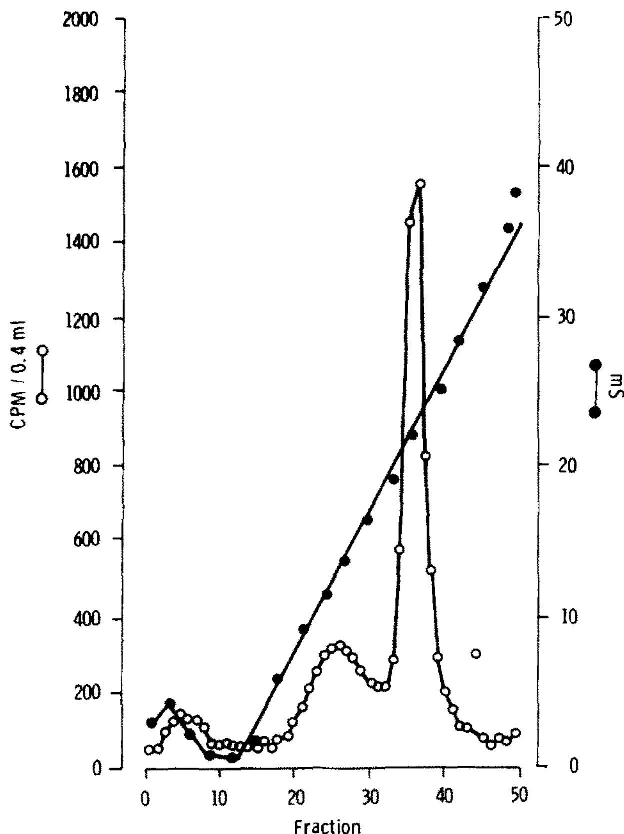

Fig. 2. DEAE chromatography of unactivated glucocorticoid receptor that had been exposed to KCl and molecular sieve chromatography. AtT-20 cells were incubated in media for 3 h at 0 with 10 nM tritiated triamcinolone acetonide. Thereupon, they were gathered, washed in Buffer I and then dounced in Buffer III. High speed cytosol was prepared and it was made 0.3 M in KCl and allowed to stand at 0°C for 30 min. One ml was placed on a Sephadex G-25 column and eluted with Buffer V. The radioactivity which came out in the exclusion volume was pooled and placed on a DEAE column and eluted.

intact cells with molybdate-free buffer led to a decrease in molybdate's protective effect, suggesting that the ion was freely diffusible and the receptor was not permanently stabilized.

DISCUSSION

We wanted to evaluate whether molybdate would alter *in vivo* nuclear translocation so that we could determine whether this change had a predictable effect on glucocorticoid action. Cold exposure is the classic means of inhibiting nuclear translocation and it is clearly effective in the AtT-20 cell (Table 1). Unfortunately, cells do not live long at this temperature. Molybdate appeared to be a promising agent; it is active *in vitro* at very low concentrations (0.5–1.0 mM) [8] and we found that these low concentrations neither killed cells nor inhibited cell growth.

When molybdate was evaluated for its ability to influence *in vivo* nuclear translocation in the AtT-20 cell, however, it was found to be inactive, even at high concentrations. The most straightforward conclusion is that this agent does not affect *in vivo* nuclear transformation. However, before this could be accepted, it had to be demonstrated that the agent entered the cell. Two pieces of evidence indicate that it did. First, molybdate inhibited cell growth and thymidine incorporation at these high concentrations suggesting that it entered the cell and interfered with some aspect of metabolism. Second, the cytosol prepared from cells which had been incubated *in vivo* with 20 mM molybdate was able to resist *in vitro* transformation (Fig. 3). Although these two pieces of evidence are suggestive, they do not unequivocably prove that molybdate entered the cell; it could be that molybdate exerted these effects through interactions at the cell membrane. Others have faced a similar question. Naray for example, analyzed the effect of molybdate on intact cell and broken cell phosphatase activity and also concluded that molybdate enters cells when present at 10 mM [9]. Hence several independent lines suggest molybdate does enter the cell.

We conclude from these studies that molybdate does not alter the magnitude of nuclear translocation nor inhibit receptor transformation in the intact, viable AtT-20 cell. Although at first this finding may seem surprising because of the wealth of *in vitro* data, these results actually are similar to those of others who studied the effects of this agent *in vivo*. Wheeler *et al.* [10] for example, studied the effect of molybdate on *in vivo* and *in vitro* receptor "activation" (in this case the term "activation" is used to describe the process that allows the receptor to convert from a non-steroid binding form to one that can bind steroids—it is not the same process as that involved in transformation.) They report that whereas *in vitro* molybdate at 0.5–1.0 mM clearly prevents receptor inactivation, *in vivo* this agent was totally ineffective—even at a concentration of 3 mM. Most importantly, Naray [9] noted, as in this report, that 10 mM

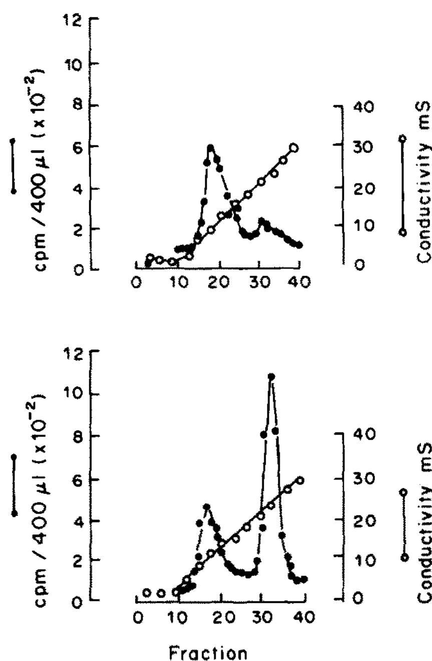

Fig. 3. Effect of having incubated AtT-20 cells *in vivo* with molybdate on subsequent *in vitro* activation. AtT-20 cells at 10^7/ml were suspended in growth media that either did not (upper panel) or did (lower panel) contain 20 mM sodium molybdate. After 30 min at 37°C 20 ml aliquots of the cells were pelleted by gentle centrifugation, quickly washed with 1 ml of ice-cold Buffer I and repelleted. These cells were resuspended in 2 ml of Buffer II and lysed through the use of a metal dounce. The homogenates were centrifuged (5 min, 800 *g*, 5°C), made 10 nM in tritiated triamcinolone acetonide and incubated in an ice bath. After 2 h the samples were warmed to 30°C for 30 min and then recooled. One ml aliquots of each were chromatographed on DEAE cellulose.

molybdate was ineffective at inhibiting *in vivo* glucocorticoid receptor translocation in the intact lymphocyte.

Hence, it is concluded that the failure of molybdate to influence transformation *in vivo* suggests either that the process of transformation that takes place in intact viable cells is not the same as that studied *in vitro* using broken cell preparations, or that molybdate, although able to enter cells and influence cellular events, is not able to interact with the receptor.

Acknowledgements—This research was supported by NIH grant AM31560. The author wishes to thank Mrs Mary Rudis and Mrs Janelle Williams for their careful technical assistance, Ms Jodi Scallan and Ms Lynne Hull for their secretarial assistance.

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