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Pyridoxal Phosphate Induced Alterations in Glucocorticoid Receptor Conformation†

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ABSTRACT: The molecular properties of dexamethasone–receptor complexes from rat thymocytes are markedly affected by pyridoxal phosphate as shown by both sucrose density gradient centrifugation and molecular exclusion gel chromatography. In the absence of added pyridoxal phosphate, unactivated cytoplasmic receptor complexes sediment as 7–8S and 2.9S species, whereas activated cytoplasmic receptor complexes sediment as 4–5S and 2.9S species. When unactivated cytoplasmic receptor complexes are exposed to pyridoxal phosphate either before or during sucrose density gradient centrifugation, the sedimentation profile is converted to one that is identical with that found for activated receptor; i.e., the 7–8S form is apparently converted to a 4–5S form. Dexamethasone receptors extracted from rat thymocyte nuclei with pyridoxal phosphate sediment as a single 3.5S peak. Findings consistent with the above are made by using gel filtration techniques. After treatment with pyridoxal phosphate followed by reduction with sodium borohydride, all cytoplasmic and nuclear dexamethasone receptors are converted to a single 2.9S form. The 2.9S form is insensitive to the presence of 0.4 M KCl during centrifugation. These observations suggest that there is an irreversible, covalent modification of dexamethasone–receptor complexes by pyridoxal phosphate and sodium borohydride. Presumably this modification occurs at a lysine residue(s) on the various forms of the receptor and causes either conformational changes in the receptors or the expression of a subunit that is common to and characteristic of all of the forms of the nuclear and cytoplasmic dexamethasone receptors found in rat thymocytes. The 2.9S forms of the dexamethasone receptors produced by treatment with pyridoxal phosphate and sodium borohydride appear to be similar in size with those produced by treatment of thymocyte cytosols with Ca^{2+}.

Current thinking on the mechanism of steroid hormone action is that the hormone enters a target cell and associates with cytoplasmic receptors. Subsequent translocation of the cytoplasmic steroid–receptor complex to the nucleus requires activation which is assumed to be accompanied by a conformational change. In vitro, the conversion of unactivated cytosolic steroid–receptor complexes can be accomplished by warming cytosolic extracts to physiological temperatures (Munck & Leung, 1977; Wira & Munck, 1974). Turnell et al. (1974) have shown by sucrose density gradient centrifugation in low-salt buffer that there are two forms of glucocorticoid receptors in cytosol from rat thymocytes which have sedimentation coefficients of approximately 7 S and 3.5 S. Studies on the molecular properties of glucocorticoid receptors from other tissues, e.g., fibroblasts (Middlebrook & Aronow, 1977) and liver (Koblinsky et al., 1972; Beato & Feigelson, 1972; Wrange & Gustafsson, 1978), indicate single ~7S receptor forms which can be converted to smaller ~4S forms by 0.4 M KCl. Even smaller forms (~3 S) of glucocorticoid receptors have been produced by limited trypsin digestion (Wrange & Gustafsson, 1978) and treatment with calcium chloride (Sherman et al., 1978). The relation between receptor size and nuclear binding is not clear at the present time.

Vitamin B_6 is an essential cofactor for a large variety of enzymes that are concerned with the intermediary metabolism of amino acids (Snell, 1958; Braunstein, 1960). Very recently, evidence has appeared suggesting that pyridoxal phosphate,

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the active form of vitamin B_6, may be directly involved in steroid hormone action. For example, Cake et al. (1978) have found that pyridoxal phosphate inhibits the binding of activated dexamethasone–receptor complexes to DNA–cellulose in vitro. Also, Nishigori et al. (1978) have shown that the interaction of an activated avian progesterone–receptor complex with ATP–Sepharose can be efficiently reversed in a specific manner by pyridoxal phosphate.

Recent studies in our laboratories have shown that pyridoxal phosphate effectively extracts dexamethasone–receptor complexes from nuclei of rat thymocytes which had been previously equilibrated with [^{3}H]dexamethasone. The extraction of nuclear dexamethasone receptor by pyridoxal phosphate is quite specific. On an equimolar basis, pyridoxal phosphate is considerably more effective than pyridoxal; pyridoxine, pyridoxamine phosphate, and 5-deoxypyridoxal are ineffective. The ability of pyridoxal phosphate to extract thymocyte nuclear dexamethasone receptor is dependent on the C4′-carboxaldehyde group of the vitamin since extraction of nuclear receptor is inhibited by carbonyl trapping reagents such as hydroxylamine and semicarbazide. Preliminary characterization of the complex extracted from nuclei by pyridoxal phosphate indicates that the steroid remains associated with a macromolecule which is about the size of myoglobin (Cidlowski & Thanassi, 1978).

We have analyzed by sucrose density gradient ultracentrifugation and gel filtration the products which result from the interaction of pyridoxal phosphate with unactivated cytoplasmic, activated cytoplasmic, and nuclear dexamethasone receptors. We present data which indicate that pyridoxal phosphate can be covalently linked to any of these forms of glucocorticoid receptors by reduction with sodium borohydride which in turn leads to the production of a 2.9S form of the glucocorticoid receptor. The sedimentation coefficient of this form of glucocorticoid receptor is insensitive to alterations in KCl concentration or hydroxylamine and is common to all forms of glucocorticoid receptors that we have studied. Moreover, it appears to be similar if not identical in size with a glucocorticoid receptor form obtained upon treatment of cytosol with calcium chloride.

Methods

General. Thymus tissue was obtained from 100 to 150 g male Sprague Dawley rats that were adrenalectomized 6–8 days prior to sacrifice. The animals were maintained on normal rat chow (Purina) and 0.85% NaCl. Cell suspensions were prepared from thymus tissue in Krebs–Ringer bicarbonate glucose buffer (KRBG) as described by Munck (1968). Hormone-binding assays were conducted at cell concentrations ranging from 5×10^8 to 1×10^9 per mL. Stock solutions of unlabeled dexamethasone (Steraloids) were prepared fresh weekly at a concentration of 1×10^{-4} M in KRBG buffer. [6,7-^{3}H]Dexamethasone (33.0 Ci/mmol) obtained from New England Nuclear was stored in benzene/ethanol. Aliquots were placed in glass incubation vials and the organic solvents evaporated before the addition of cell suspensions. Pyridoxal phosphate (Aldrich) was prepared fresh daily in 1.5 mM $MgCl_2$, and the pH of the solution was adjusted to 7.0. All manipulations which involved pyridoxal phosphate were performed in subdued light. Other chemicals were reagent grade and were obtained from Sigma or Fischer Scientific.

Preparation of Unactivated, Activated, and Nuclear Dexamethasone–Receptor Complexes. Unactivated cytoplasmic dexamethasone receptors are defined as those steroid hormone–receptor complexes which will not bind to nuclei or DNA–cellulose during incubations at 0 °C, whereas activated cytoplasmic dexamethasone receptors are those which will associate with either of these acceptors during 0 °C incubations. The methodology for production of unactivated and activated dexamethasone–receptor complexes has been outlined in detail elsewhere (Cidlowski & Munck, 1978). Briefly, the preparation of both unactivated and activated cytoplasmic dexamethasone–receptor complexes is initiated by the incubation of 1–2 mL of thymocyte cell suspension with 2×10^{-8} M [^{3}H]dexamethasone in the presence or absence of 2×10^{-6} M unlabeled dexamethasone for 2 h at 0 °C. Following centrifugation at 500*g*, the supernatant is removed and 6 volumes of ice-cold 1.5 mM $MgCl_2$ is added to the cell pellet to lyse the cells. DNA and broken nuclei are removed by centrifugation at 1500*g* for 10 min. The supernatant (cytosol) contains cytoplasmic dexamethasone receptors, >95% of which are in the unactivated form. Activated receptors are formed from this preparation by warming the cytosol solution containing unactivated steroid–receptor complexes for 15 min at 25 °C. This procedure results in maximal receptor activation with a 25–35% loss in receptor binding activity, probably due to steroid dissociation from receptor (Cidlowski & Munck, 1978).

Treatment of Cytoplasmic Receptors with Pyridoxal Phosphate and Extraction of Nuclear Dexamethasone Receptors with Pyridoxal Phosphate. Pyridoxal phosphate was prepared in a concentrated stock solution (50 mM, pH 7.0) in 1.5 mM $MgCl_2$ prior to use in these experiments. One or two milliliters of either activated or unactivated cytosol samples containing dexamethasone receptors were brought to a final concentration of 5 mM pyridoxal phosphate by the addition of an aliquot of stock solution. The cytosols containing pyridoxal phosphate were then incubated for 90 min at 3 °C in the dark.

Dexamethasone receptors were extracted from thymocyte nuclei with 5 mM pyridoxal phosphate, as previously described (Cidlowski & Thanassi, 1978). Essentially the nuclei from 1.0 mL of thymocyte cell suspensions were extracted with 2.0 mL of pyridoxal phosphate solution for 90 min in the dark at 3 °C, while being agitated at 60 cycles/min on a rotary shaker. After the extraction period, the nuclei and nuclear debris were sedimented by centrifugation at 1500*g* for 10 min. The supernatant "nuclear extract" was removed and a portion treated as follows. Reduction of pyridoxal phosphate to pyridoxine phosphate and protein-bound pyridoxal phosphate (presumably as phosphopyridoxyllysine) was accomplished by the addition of solid $NaBH_4$ to ice-cold cytosol samples or nuclear extracts until the characteristic yellow color of pyridoxal phosphate was blanched.

Prior to ultracentrifugation on sucrose gradients, 1.0 mL of each sample was mixed for 10 min at 3 °C with dextran-coated charcoal prepared from 1.0 mL of a 1% Norit-A/0.1% dextran suspension in 1.5 mM $MgCl_2$. The dextran-coated charcoal, containing free steroid, was then removed by centrifugation at 1500*g* for 10 min, and the supernatant was used for study.

Gel Filtration Studies. Gel filtration analyses of pyridoxal phosphate treated cytoplasmic and nuclear dexamethasone receptors were performed by using a 2 × 10 cm Sephadex G-100 column. The column was equilibrated with 1.5 mM $MgCl_2$ and had a blue dextran exclusion volume of 7.0 mL; free dexamethasone eluted after fraction 60. The extraction procedures for nuclear dexamethasone receptors and the treatment of cytoplasmic receptors with pyridoxal phosphate are provided in the legend to Figure 1. These differ from the

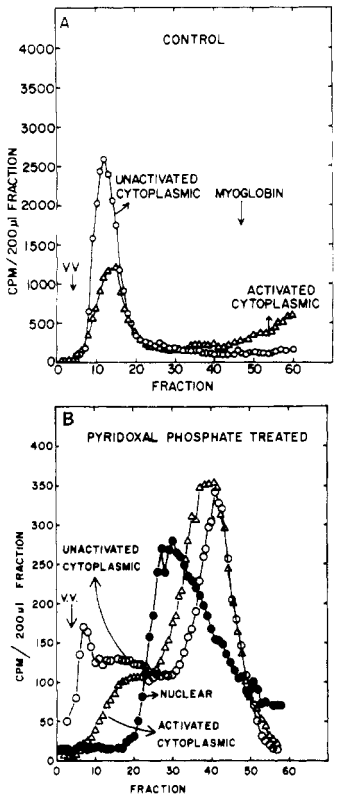

FIGURE 1: (A) Sephadex G-100 gel filtration of unactivated and activated dexamethasone–receptor complexes. Non-pyridoxal phosphate treated, activated, and unactivated dexamethasone receptors prepared as described under Methods were chromatographed, and 5-drop (200 μL) fractions of the eluate were collected into scintillation vials. (B) Sephadex G-100 gel filtration of cytoplasmic and nuclear forms of dexamethasone–receptor complexes treated with pyridoxal phosphate. Cytosols containing unactivated or activated cytoplasmic dexamethasone receptors were treated with 20 mM pyridoxal phosphate for 60 min at 3 °C prior to gel filtration. Dexamethasone–receptor complexes were extracted from nuclei of cells by incubation of the nuclei from 1.5×10^9 cells with 2.0 mL of 20 mM pyridoxal phosphate prepared in 1.5 mM $MgCl_2$ for 30 min at 3 °C. After centrifugation of the nuclear debris for 10 min at 1500*g*, a 1.0-mL sample of the supernatant was placed on the column. Five-drop fractions were collected into scintillation vials and counted for radioactivity.

conditions employed in the sucrose density gradient studies in that 20 mM pyridoxal phosphate was used and there was no reduction with $NaBH_4$.

Sucrose Density Gradient Centrifugation of Dexamethasone–Receptor Complexes. Linear 5–20% sucrose gradients (4.5 mL) were prepared from solutions of sucrose in 10 mM Tris, 1 mM Na_2EDTA, and 12 mM α-thioglycerol buffer at pH 7.5. Four-hundred microliter samples were layered on each gradient, and the tubes were centrifuged in a Beckman S.W. 50.1 rotor at 45 K for 16 h at 2 °C. Following centrifugation, the tubes were punctured in the bottom and 10-drop fractions were collected by gravity into scintillation vials. The molecular weight markers myoglobin (2.0 S) and bovine serum albumin (4.6 S) were centrifuged in companion tubes in each experiment. After the fractionation, the position of the myoglobin peak was determined at 410 nm, and the position of the bovine serum albumin (BSA) was determined by the method of Lowry et al. (1951). Steroid–receptor complex sedimentation coefficients were calculated as described by Martin & Ames (1962).

Radioactivity was measured in a Beckman LS-150 scintillation spectrometer having an efficiency of 40% for tritium. The samples were counted in 3.0 mL of PCS (Amersham/Searle)/xylenes (2:1, v:v). No significant variation in counting efficiency was observed among samples.

Results

Figure 1A provides the Sephadex G-100 gel elution profiles for unactivated and activated cytoplasmic dexamethasone receptors. Both forms of receptor elute as single peaks of macromolecular bound radioactivity between fractions 7 and 20. The molecular weight marker myoglobin (16 800) elutes in fraction 47 and free dexamethasone elutes after fraction 60. In Figure 1B are shown the profiles for unactivated and activated cytoplasmic dexamethasone receptors treated with pyridoxal phosphate as well as nuclear dexamethasone receptors extracted with pyridoxal phosphate. Treatment of unactivated receptors with pyridoxal phosphate results in a change in the elution profile, with the major portion of the macromolecular bound radioactivity appearing in fractions 30–50. However, there is still a residual peak between fractions 10 and 20 corresponding to the only peak found in unactivated, untreated receptors (Figure 1A). The apparent peak at fraction 7 most likely represents aggregation and is not seen in most experiments of this kind. Similar observations were made when activated receptors were treated with pyridoxal phosphate. Dexamethasone receptors extracted from thymocyte nuclei with pyridoxal phosphate chromatograph as a broad peak slightly larger than either of the cytoplasmic forms which were treated with pyridoxal phosphate (Figure 1B). In the experiment reported in Figure 1B, receptor associated radioactivity was 55–70% of the total radioactivity applied to the column.

These observations were confirmed and extended by experiments utilizing sucrose density gradient centrifugation. Figure 2A shows centrifugation profiles of unactivated and activated cytoplasmic dexamethasone receptors on linear 5–20% sucrose gradients. Unactivated cytoplasmic receptors show peaks of radioactivity in the 7–8S and 2.9S regions. Activated cytoplasmic receptors show peaks of radioactivity in the 4–5S and 2.9S regions. These findings are dependent upon treatment of cytosols with dextran-coated charcoal to remove free steroid prior to centrifugation. If this step is eliminated, only the heavier forms of the receptors can be seen because the 2.9S peak becomes hidden under the radioactivity contributed by free steroid. Activation of receptor by warming apparently results in a change in sedimentation of the heavier 7–8S form of the unactivated receptor to a 4–5S form. No alteration in the sedimentation coefficient of the lighter 2.9S form of the receptor occurs following activation by heating. The profiles shown in Figure 2A are representative. However, we have observed that the ratios of the 2.9S to 4–5S and 7–8S forms vary between experiments. Figure 2B shows the results of an experiment in which the unactivated and activated forms of dexamethasone receptor are centrifuged through sucrose gradients containing 1 mM pyridoxal phosphate. In contrast to centrifugation in the absence of pyridoxal phosphate, the heavier species (7–8 S) of unactivated receptor now sediments coincident with the heavier form of activated receptor, i.e., 4–5 S. These data indicate that the presence of pyridoxal phosphate during centrifugation alters the characteristics of the 7–8S form of the unactivated receptor. This treatment results in a sedimentation profile for unactivated receptors similar to that observed with heat-activated receptors which have not been treated with pyridoxal phosphate (Figure 2A).

We next studied the influence of the reducing agent sodium borohydride, $NaBH_4$, on the sedimentation behavior of pyridoxal phosphate treated cytoplasmic receptors or nuclear receptors extracted by pyridoxal phosphate. We presume that

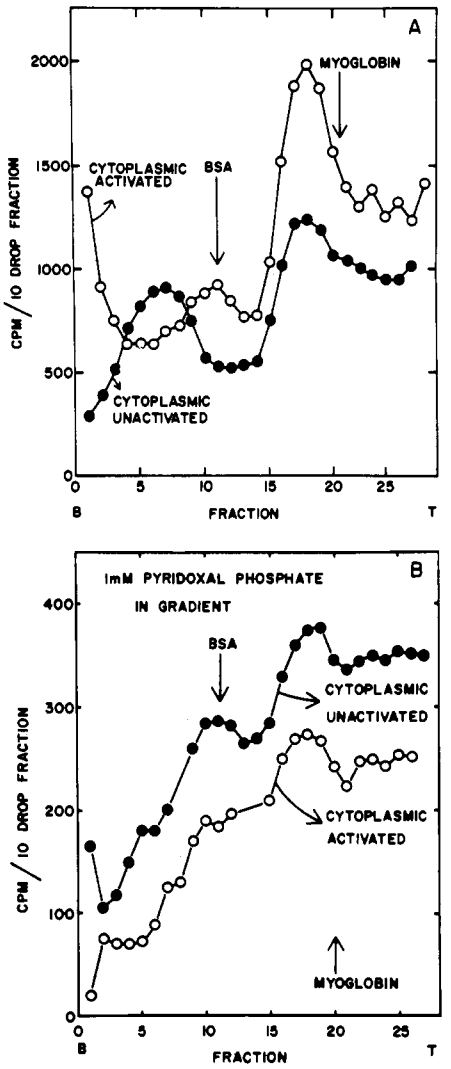

FIGURE 2: Analysis of cytoplasmic dexamethasone receptors on sucrose gradients in the presence or absence of pyridoxal phosphate. (A) Sucrose density gradient analysis of unactivated and activated cytoplasmic dexamethasone receptors from thymocytes incubated at 0 °C with 2×10^{-8} M [^{3}H]dexamethasone. Each receptor preparation was treated with dextran-coated charcoal just prior to layering on the sucrose gradient. Low-salt gradients were prepared, centrifuged, and fractionated as described under Methods. Total charcoal resistant cpm applied to the gradients were 30 450 for unactivated receptors and 22 100 for activated receptors. (B) Density gradient analysis of unactivated and activated cytoplasmic receptors in gradients containing 1 mM pyridoxal phosphate. Unactivated and activated cytoplasmic receptors were prepared as described. All receptor fractions were treated with dextran-coated charcoal just prior to layering the samples on the gradients. Total charcoal resistant cpm applied to the gradients were 7500 for the unactivated receptors and 4750 for the activated receptors. Bovine serum albumin (4.6 S) and myoglobin (2.0 S) were used as molecular weight markers in simultaneously centrifuged preparations. B denotes bottom of the gradient; T denotes top of the gradient.

$NaBH_4$ will reduce Schiff base linkages which can form between lysine residues on the receptor and pyridoxal phosphate (Fischer et al., 1958). Figure 3 shows the sedimentation pattern for unactivated cytoplasmic dexamethasone receptors either treated with 5 mM pyridoxal phosphate alone or treated with 5 mM pyridoxal phosphate followed by reduction with $NaBH_4$ prior to centrifugation on sucrose gradients that do not contain pyridoxal phosphate. Also shown are the data for pyridoxal phosphate treated cytoplasmic receptors obtained from cells that had been incubated with [^{3}H]dexamethasone plus a saturating concentration of unlabeled steroid. The elimination of binding by the addition of unlabeled steroid

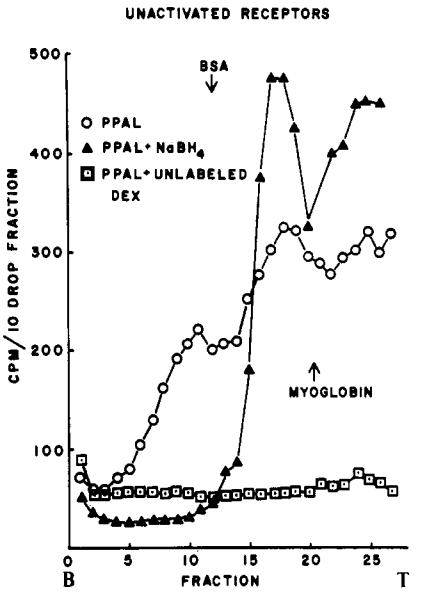

FIGURE 3: Sucrose density gradient centrifugation of pyridoxal phosphate treated, unactivated cytoplasmic dexamethasone receptors. Unactivated receptors were prepared from thymocytes incubated at 0 °C with [^{3}H]dexamethasone alone or [^{3}H]dexamethasone plus a 100-fold molar excess of unlabeled dexamethasone as described under Methods. Cytoplasmic samples were treated with 5 mM pyridoxal phosphate prepared in 1.5 mM $MgCl_2$, pH 7.0, for 90 min at 3 °C. Subsequently, $NaBH_4$ was added to one sample to reduce pyridoxal phosphate as detailed under Methods. The samples were then treated with dextran-coated charcoal, centrifuged, fractionated, and counted exactly as described under Methods. Total charcoal resistant cpm applied to gradients were 5895 in the pyridoxal phosphate treated, unactivated receptors and 4979 in the pyridoxal phosphate treated plus $NaBH_4$ reduced unactivated receptors.

indicates that we are monitoring saturable receptor binding in both 4–5S and 2.9S forms. Treatment of unactivated receptor with pyridoxal phosphate results in a bimodal sucrose density gradient profile identical with that shown in Figure 2B where unactivated receptors are sedimented in gradients which contain 1 mM pyridoxal phosphate. Thus, pretreatment of unactivated cytoplasmic receptor with 5 mM pyridoxal phosphate or centrifugtion of untreated receptors through sucrose gradients containing 1 mM pyridoxal phosphate give identical results. Reduction with $NaBH_4$ of the pyridoxal phosphate treated unactivated receptors, however, results in a marked change in the receptor sedimentation profile, with all of the receptor now sedimenting in the low molecular weight 2.9S form. $NaBH_4$ treatment of unactivated receptors not exposed to pyridoxal phosphate does not lead to any alteration in the characteristic sedimentation profile for unactivated receptors (data not shown).

Figure 4 shows the sedimentation profile for activated cytoplasmic receptors which were treated with pyridoxal phosphate alone, or treated with pyridoxal phosphate and $NaBH_4$. Treatment of activated receptor with pyridoxal phosphate alone results in a bimodal receptor sedimentation profile with peaks of radioactivity at both 4–5 S and 2.9 S. Reduction of pyridoxal phosphate treated dexamethasone receptors with $NaBH_4$ results in a unimodal receptor sedimentation profile with all of the bound hormone–receptor complex sedimenting as a 2.9S species. Thus, when either unactivated (Figure 3) or activated (Figure 4) pyridoxal phosphate treated cytoplasmic receptors are reduced with $NaBH_4$, a similar 2.9S unimodal sucrose gradient profile is observed.

Figure 5 provides the sedimentation profile for dexamethasone receptors which have been extracted by pyridoxal

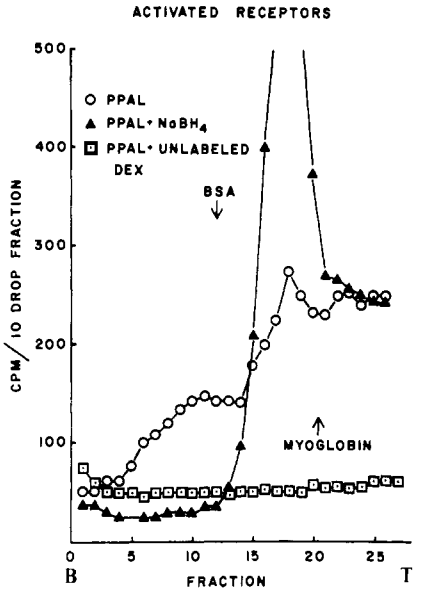

FIGURE 4: Sucrose density gradient centrifugation of pyridoxal phosphate treated, activated cytoplasmic dexamethasone receptors. Heat-activated cytoplasmic receptors were prepared from thymocytes incubated at 0 °C with 2×10^{-8} M [^{3}H]dexamethasone alone or [^{3}H]dexamethasone plus 2×10^{-6} M unlabeled dexamethasone. The procedures for receptor activation and their subsequent treatment with pyridoxal phosphate alone or pyridoxal phosphate followed by $NaBH_4$ have been previously described. Total charcoal resistant cpm applied to the gradients were 4320 in the pyridoxal phosphate treated, activated receptors and 4255 in the pyridoxal phosphate plus $NaBH_4$-treated, activated receptors.

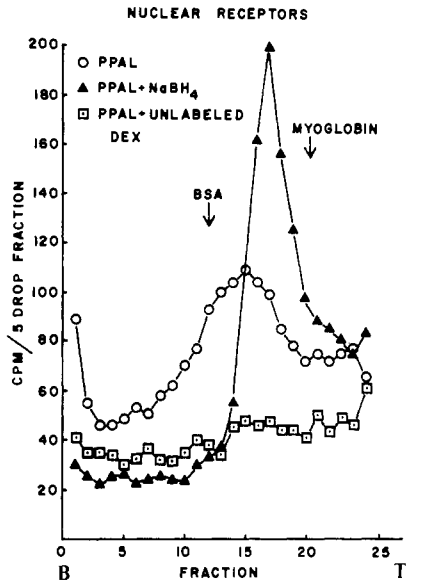

FIGURE 5: Sucrose density gradient centrifugation of nuclear dexamethasone–receptor complexes extracted by pyridoxal phosphate. Dexamethasone–receptor complexes were extracted from the nuclei of thymocytes incubated with 2×10^{-8} M [^{3}H]dexamethasone alone or 2×10^{-8} M [^{3}H]dexamethasone plus 2×10^{-6} M unlabeled dexamethasone. One-half of the nuclear extract was treated further with $NaBH_4$. Both samples were treated with dextran-coated charcoal prior to centrifugation. Total charcoal resistant cpm applied to the gradients were 1857 for the nuclear receptors extracted by pyridoxal phosphate and 1545 for the nuclear receptors extracted by pyridoxal phosphate and reduced with $NaBH_4$.

phosphate from nuclei of whole thymocytes (Cidlowski & Thanassi, 1978). Such receptors have a unimodal sedimentation profile on sucrose gradients with the major peak of radioactivity observed at appproximately 3.5 S. These results are consistent with our findings by using gel filtration

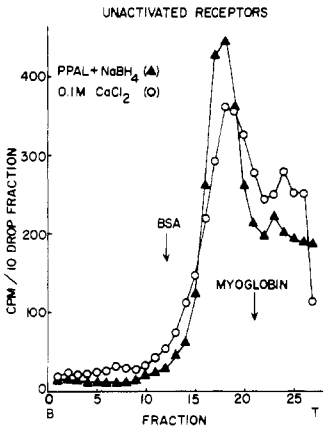

FIGURE 6: Sucrose density gradient centrifugation of unactivated dexamethasone receptors treated with calcium chloride or with pyridoxal phosphate plus sodium borohydride. Unactivated dexamethasone receptors were prepared from thymocytes incubated with 2×10^{-8} M [^{3}H]dexamethasone at 0 °C as described under Methods. The receptors were either treated with pyridoxal phosphate plus sodium borohydride as described in the legend to Figure 3 or were treated with 0.1 M $CaCl_2$ for 90 min at 0 °C. Both preparations were then treated with dextran-coated charcoal prior to application to the gradient. Total charcoal resistant cpm applied were 3844 for the pyridoxal phosphate plus sodium borohydride reduced unactivated receptors and 4006 for the $CaCl_2$-treated receptors.

(Figure 1) which indicated that nuclear receptors extracted by pyridoxal phosphate exist in only one form which is slightly larger than the 2.9S forms of either unactivated or activated cytoplasmic receptors. When these nuclear receptor complexes are subsequently reduced with $NaBH_4$, the sedimentation profile changes with all of the radioactivity found in a 2.9 S form.

Several investigators (Turnell et al., 1974; Beato & Feigelson, 1972) have shown that cytoplasmic glucocorticoid receptors are sensitive to the presence of 0.3–0.4 M KCl during centrifugation. Their results show that high salt causes conversion of a heavier 7–8S form of cytoplasmic receptors to smaller forms (3–4 S). In our experiments, the presence of 0.4 KCl during centrifugation causes the apparent conversion of either 7–8S or 4–5S cytoplasmic receptors to 2.9S receptors. Because of the report by Sherman et al. (1978) that the presence of 0.1 M $CaCl_2$ in cytosolic preparations leads to the production of a low molecular weight "meroreceptor", we have investigated the effect of calcium chloride in our system. As shown in Figure 6, the presence of 0.1 M $CaCl_2$ for 90 min in the incubation mixtures prior to treatment with dextran-coated charcoal causes the formation of a 2.9S cytoplasmic receptor form. Therefore, three agents, KCl, $CaCl_2$, and pyridoxal phosphate, can lead to the production of 2.9S forms of dexamethasone receptors from rat thymocytes. On a molar basis, pyridoxal phosphate is at least 1–2 orders of magnitude more effective than the inorganic salts in effecting this conversion.

Discussion

The possibility of an interaction between steroid hormone receptors and pyridoxal phosphate has been suggested by several studies. Three lines of evidence indicate that pyridoxal phosphate may directly affect steroid–receptor complexes. Cake et al. (1978) have observed that addition of pyridoxal phosphate to cytosols inhibits the binding of activated dexamethasone receptors to DNA–cellulose in vitro under low ionic strength conditions. Nishigori et al. (1978) reported that binding of activated avian progesterone–receptor complexes to ATP–Sepharose can be inhibited by pyridoxal phosphate.

Our studies have shown that pyridoxal phosphate can specifically and effectively extract dexamethasone–receptor complexes from nuclei of thymocytes previously equilibrated with [^{3}H]dexamethasone (Cidlowski & Thanassi, 1978). These studies also suggested that the product extracted from nuclei was a small form of dexamethasone–receptor complex similar in size to meroreceptors recently described for glucocorticoids which are produced by the addition of exogenous calcium ions (Sherman et al., 1978). These observations prompted us to study the effects of exogenous pyridoxal phosphate on the physical properties of both unactivated and activated cytoplasmic receptors as well as nuclear glucocorticoid–receptor complexes from rat thymocytes.

Both Turnell et al. (1972) and we (Figure 2B) have found that unactivated glucocorticoid receptors from rat thymocytes sediment as two forms (2.9 S, 7–8 S) on low-salt sucrose gradients. After warming of these receptors to 25 °C for 15 min, conditions which endow the receptors with the capability of binding to nuclei or DNA–cellulose (Cidlowski & Munck, 1978), an alteration in receptor sedimentation behavior occurs such that the heavier 7–8S form of unactivated receptor is converted to a form sedimenting at 4–5 S. These data show that warming affects the physical properties of the 7–8S form of glucocorticoid–receptor complexes. The 7–8S form of estrogen receptor (Giannopoulos & Gorski, 1971) has been suggested to be an aggregate. We have no information on this point for thymocyte dexamethasone receptors except that we observe a 7–8 S species when cytosols are prepared with either 1.5 mM $MgCl_2$ or with 1.5 mM $MgCl_2$ plus 0.15 M KCl. Only the sedimentation of the heavier form of unactivated dexamethasone–receptor complex is affected by the presence of 1 mM pyridoxal phosphate (Figure 2B). In the presence of 1 mM pyridoxal phosphate, both activated and unactivated cytoplasmic receptors appear as 4–5 S and 2.9 S forms. These two forms of receptor, 4–5 S and 2.9 S, are similar in size to those found on gel filtration following treatment of activated and unactivated cytoplasmic receptors with pyridoxal phosphate (Figure 1B). These experiments demonstrate that both heat activation and pyridoxal phosphate alter the sedimentation behavior of the higher molecular weight form (7–8 S) of unactivated dexamethasone–receptor complexes. These data, in combination with the sodium borohydride experiments discussed below, suggest that a direct interaction occurs between pyridoxal phosphate and dexamethasone receptor. At the present time, we have no information on whether pyridoxal phosphate is associated with the 2.9 S form of receptor or releases the 2.9 S form of receptor following interaction with larger forms of receptor. We are addressing this question using radioactive pyridoxal phosphate.

To further characterize the interaction of pyridoxal phosphate with glucocorticoid receptors, we studied the effects of sodium borohydride. Presumably the vitamin is bound to receptor as a Schiff base involving the ϵ-amino group of lysine residues. Reduction of such Schiff bases with $NaBH_4$ results in the irreversible covalent attachment of pyridoxal phosphate. The results in Figure 3 and 4 clearly show that reduction of pyridoxal phosphate treated unactivated and activated dexamethasone–receptor complexes with $NaBH_4$ results in sucrose density gradient profiles containing only a 2.9S receptor form. These data show that the covalent attachment of pyridoxal phosphate has dramatic effects on the larger forms of steroid–receptor complexes in both unactivated and activated cytoplasmic receptor preparations. Nuclear dexamethasone receptors extracted by pyridoxal phosphate as described previously (Cidlowski & Thanassi, 1978) sediment as a single 3.5S form which also upon reduction with $NaBH_4$ converts to a 2.9S species. Thus, the 2.9S receptor form is found in untreated unactivated cytosols and untreated activated cytosols either in the presence or absence of pyridoxal phosphate. It is exclusively found when both activated and unactivated cytoplasmic receptors and nuclear receptors are treated with pyridoxal phosphate and $NaBH_4$. It is clear, therefore, that pyridoxal phosphate interacts with cytoplasmic and nuclear glucocorticoid receptors. Such interaction leads to changes in receptor sedimentation coefficients that become irreversible after reduction with $NaBH_4$. The observation of endogenous 2.9S receptors suggests that either pyridoxal phosphate or a molecule having similar action may be bound to the receptor in the intact cell. Our finding that $NaBH_4$ treatment of unactivated or activated receptors in the absence of added pyridoxal phosphate does not alter receptor sedimentation profiles from animals maintained on vitamin B_6 sufficient diets suggests that endogenous pyridoxal phosphate levels are not saturating with respect to receptors or that the vitamin may be dissociated from receptor during its preparation. Alternatively, as discussed above, pyridoxal phosphate may not be associated with the steroid binding portion of the receptor so that reduction of untreated endogenous receptors with $NaBH_4$ will be without effect.

Several investigators (Sherman et al., 1978; Puca et al., 1971, 1972; Wrange & Gustafsson, 1978; Carlson et al., 1977) have produced small forms of steroid–receptor complexes by either Ca^{2+} treatment or protease digestion which are similar in size to those which we have described here for pyridoxal phosphate treated receptors. The 2.9S form of the glucocorticoid receptor that we observe is an endogenous component of both activated and unactivated cytosols, and is the sole product observed on sucrose gradients after pyridoxal phosphate treatment and $NaBH_4$ reduction. It appears, therefore, that $NaBH_4$ reduction of pyridoxal phosphate treated receptors serves to lock the receptor into its 2.9S form. In an effort to relate our findings to those of others, we examined the effects of 0.4 M KCl during centrifugation and 0.1 M $CaCl_2$ treatment prior to centrifugation on the sedimentation properties of rat thymocyte glucocorticoid receptors. Both of these treatments result in the formation of only 2.9S species. Thus, three very different reagents are capable of producing an apparently common form of receptor. However, with reference to the mechanism of action of pyridoxal phosphate on glucocorticoid receptor properties, we have evidence that the pyridoxal phosphate induced formation of the 2.9S form of receptor is different from that which involves Ca^{2+}-dependent proteolysis as reported by Sherman et al. (1978). Thus we have found that Ca^{2+}-induced meroreceptor formation is effectively inhibited by 10 mM *N*-ethylmaleimide, whereas formation of the 2.9S species by pyridoxal phosphate and $NaBH_4$ treatment is not inhibited by 10 mM *N*-ethylmaleimide. These observations suggest that pyridoxal phosphate either induces changes in receptor conformation or causes disaggregation rather than increasing the receptor's susceptibility to proteolysis. It is worth mentioning in this context that we have observed pyridoxal phosphate effects on receptor properties at a concentration of 1 mM; this is the lowest concentration we have tested. Although the physiological implications of these findings are not clear at the present time, pyridoxal phosphate may conceivably be involved in the regulation of steroid hormone action. Such regulation could involve phosphorylation and dephosphorylation of the vitamin.

In conclusion, our experiments have shown that pyridoxal phosphate can interact with unactivated, activated, and nuclear

glucocorticoid receptors, presumably via Schiff base formation with ε-amino groups of lysine residues. Following reduction with $NaBH_4$, which itself has no effect on receptor, a small 2.9S form of glucocorticoid receptor is produced.

We have observed that the 2.9S form of receptor obtained after pyridoxal phosphate treatment and $NaBH_4$ reduction has considerable stability in vitro. This should allow us to better analyze the properties of glucocorticoid receptors which until now have been difficult because of receptor instability.

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Effects of Thio-Group Modification and Ca^{2+} on Agonist-Specific State Transitions of a Central Nicotinic Acetylcholine Receptor†

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ABSTRACT: Agonist-binding affinities of central nervous system nicotinic acetylcholine receptors (nAcChR) are sensitive to the duration of exposure to agonist. These agonist-induced changes in receptor state may be mimicked by appropriate modification of receptor thio groups and/or by manipulation of solvent ionic composition. In the absence of Ca^{2+}, the concentration of acetylcholine (AcCh) necessary to prevent half of specific ^3H-labeled α-bungarotoxin binding is ~1 mM for nAcChR treated with dithiothreitol (DTT) or DTT–*N*-ethylmaleimide (low-affinity states) and ~40 μM for nAcChR treated with DTT–5,5′-dithiobis(2-nitrobenzoic acid) or for native nAcChR pretreated with AcCh (high-affinity states). Addition of Ca^{2+} results in an increase in the effectiveness of AcCh toward blocking toxin binding. None of these treatments alters toxin or antagonist binding nor are there observed differences in Hill numbers for agonist binding. Agonists competitively inhibit toxin binding to low-affinity states, but noncompetitive inhibition is observed for binding to high-affinity states. Values of AcCh dissociation constants estimated from these data fall within the range of values determined physiologically with nAcChR from other systems. The data indicate that the redox state of brain nAcChR thio groups and Ca^{2+} may mediate physiologically important changes in the receptor state during activation and desensitization.

The selective response of neurotransmitter receptors to agonist is fundamental to their physiological role in regulation of nerve impulse initiation. Recently, reports have appeared describing agonist-specific changes in receptor affinity for cholinergic agonists. These changes have been detected by inhibition of curaremimetic neurotoxin binding to peripheral (Weiland et al., 1976, 1977; Weber et al., 1975; Colquhoun & Rang, 1976; Barrantes, 1976, 1978; Lee et al., 1977; Quast et al., 1978) and central [Lukas(iewicz) & Bennett, 1978a,b] nAcChR.[1,2] These results suggest that nAcChR selectively

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[1] Abbreviations used: nAcChR, nicotinic acetylcholine receptor(s); CNS, central nervous system; DTT, dithiothreitol; AcCh, acetylcholine; MalNEt, *N*-ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); α-Bgt, α-bungarotoxin; [^3H]-α-Bgt, ^3H-labeled α-bungarotoxin; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IC_{50}, concentration of competitor required to block 50% specific [^3H]-α-Bgt binding; K_D^{app}, apparent dissociation constant for [^3H]-α-Bgt binding; n_H, Hill number for competitor binding to nAChR.

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