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The Glucocorticoid Receptor of the Neural Retina of the Chick Embryo: Gradual Changes in Charge of the Glucocorticoid Receptor Complex During Transformation, Detected by Electrofocusing

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THE GLUCOCORTICOID RECEPTOR OF THE NEURAL RETINA OF THE CHICK EMBRYO: GRADUAL CHANGES IN CHARGE OF THE GLUCOCORTICOID RECEPTOR COMPLEX DURING TRANSFORMATION, DETECTED BY ELECTROFOCUSING.

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ABSTRACT

Two main difficulties impede the monitoring of the changes in the surface charge of the triamcinolone acetonide-glucocorticoid-receptor (TA-GR) complex concomitant with GR-transformation by electrofocusing (EF). One difficulty relates to the high sensitivity of the nonactivated [^{3}H]TA-GR complex to temperature. The second difficulty relates to the transforming effects of basic carrier ampholytes, pI range 8-10 (basic SCAMs) on GR. Performing EF at strictly controlled cold temperature, and omitting the use of basic SCAMS revealed that the nontransformed molybdate-stabilized TA-GR complexes in the cytosol of the neural retina consist of a homogeneously charged GR population exhibiting an apparent pI (pI') value of 5.0 $\pm$ 0.2 (contrary to the previously reported heterogeneity of GR in 'nonactivated' state[1]). Exposure of the [^{3}H]TA-GR to activation by physiological temperatures, or to activation by 0.4M KCl in the cold, (in the presence of PMSF, which inhibits proteolytic degradation of GR in the neural retina[2]) transformed the acidic

nonactivated GR complex (designated TA-GR I) to more basic GR species: TA-GR II with a pI' of 5.6 ± 0.2 and TA-GR III with a pI' of 6.2 ± 0.1. TA-GR II appeared as a minor component, and TA-GR III appeared as the major form in the activated state. Molybdate inhibited the formation of TA-GR III induced by 0.4M KCl, while it augmented TA-GR II. The formation of TA-GR II from molybdate-stabilized TA-GR I was enhanced by cytosol dilution. The increase in the amount of TA-GR II in molybdate-stabilized cytosol correlated with the decrease in the amount of TA-GR I and the absence of III, suggesting that TA-GR II represents an intermediate state of GR transformation. The suppression of TA-GR III by molybdate is reversible at 30°C. Inhibitors of proteases, leupeptin, antipain and PMSF, caused a slight decrease in the pI' value of TA-GR II found in the activated cytosol, (5.4 instead of 5.7), but did not change the heterogeneity of GR species found in the activated state. The omission of basic SCAMs in this study reduced the previously reported pI' values[1] of TA-GR species which had been artifactually increased by basic SCAMs. The pI' values reported in this study were confirmed by replacing SCAMs with simple buffers as carrier constituents, and appear to reflect the authentic surface net charge of TA-GR. EF analysis of [^{3}H]TA-GR complexes that were formed in the intact tissue at 0°C (cell-bound [^{3}H]TA-GR) confirmed the charge homogeneity of the nonactivated GR complexes in the nonactivated cytosol but showed that the cell-bound nonactivated [^{3}H]TA-GR is more acidic than the TA-GR I formed under cell-free conditions, exhibiting a pI' value of 4.2 ± 0.1. These results are consistent with the hypothesis that GR transformation is a multistage process characterized by a progressive decrease in the negative net charge of the GR complex. The nature of the changes in negative net charge is discussed elsewhere[2] in relation to the changes in size of the GR complex occurring in the process of GR transformation.

INTRODUCTION

The glucocorticoid receptors (GRs) in hormone-free tissue are found in the cytosol of the target cells[3,4]. The binding of the glucocorticoid hormone (GC) to its cognate receptors, under physiological conditions, triggers the transformation of hormone-receptor complexes to transcription regulating factors that translocate to the nucleus, bind to nuclear acceptor sites and modulate tissue-specific gene transcription [5,6]. The cytosolic, nontransformed GR has been characterized in various GC-target tissues as an oligomer with a molecular mass of 320-350 kilodaltons (kDa)[7]. The activated GR, distinguished by its ability to bind to nuclei, DNA or polyanion matrices[8,9], has been characterized as a species of 90-110 kDa (reviewed in [7]). The acquired DNA-binding activity of the active GR correlates with the decrease in negative net charge of the receptor concomitant with activation, as evidenced by ion-exchange chromatography[9,10]. However, this change in charge could not be detected in various GC-target cells when studied by electrofocusing (EF). Acidic and more basic GR species were found in both nonactivated and activated cytosols in a similar distribution[11,12]. Furthermore, in some GR systems in which a decrease in the negative net charge following activation has been detected, the apparent pI (pI') values of the activated GR species were in the range of 7.3 to 8.0[1,13] which is much more basic than the pI' values of the most basic GR species found in many other GC-target tissues[11,12,14].

It has been suggested that the heterogeneity in charge of GR species found in the EF of the nonactivated cytosol reflects the heterogeneity of the GR system[12]. Others ascribed the heterogeneity to proteolytic degradation of GR[14,15]. Studies on the neural retina raised the possibility that the heterogeneity may reflex GR in various states of GR transformation[1], suggesting that GR transformation is a multistage process. This study provides experimental support for this hypothesis. It

is shown that a) the variously charged species of GR arise under conditions which presumably inhibit the proteolytic degradation of GR[2]; and b) the heterogeneity of GR found in the nonactivated cytosol is an artifact introduced by the EF procedures. This study clearly demonstrates that the GRs in the nonactivated cytosol are homogeneously charged acidic complexes. Establishing the experimental conditions which prevent uncontrolled transformation of GR facilitated the monitoring of changes in the charge of the GR complex during the process of transformation, as reported in this study.

Attempts to relate the variously charged species of GR resolved by EF to the forms of GR of various sizes detected in the process of GR transformation are reported elsewhere[2].

MATERIALS AND METHODS

1) Buffers.

The following homogenization buffers were used: Buffer A: 10mM Tricine, 1.5mM EDTA, 12mM thioglycerol in 10% glycerol (v/v), pH 7.25 at 25°C (TETG). Buffer B: buffer A containing in addition 20mM sodium molybdate (TETGM). Buffer C: buffer A containing in addition 1mM phenylmethylsulfonylfluoride (PMSF) (TETG-P). Buffer D: buffer B containing 1mM PMSF (TETGM-P). The presence of PMSF in the homogenization buffers inhibits proteolytic degradation of GR in the neural retina[2]. All chemical reagents used were of analytical grade.

2) Preparation of [^{3}H]TA-GR in Cell-Free Cytosol.

Neural retinas of 14-day-old chick embryos were homogenized in buffer containing a saturating dose of [^{3}H]TA (2.7×10^{-8}M, 43 Ci/mmole, NEN), using a Dounce tissue homogenizer with the fine clearance pestle. Routinely, 50μl buffer per retina (~ 3 mg protein/neural retina) were used. The homogenate was centrifuged at 226,000 g for 60 min at 2°C. The supernatant cytosol was allowed to equilibrate with [^{3}H]TA for 2h in an ice-water shaking bath.

3) Preparation of [^{3}H]TA-GR in the Intact Tissue (Cell-Bound TA-GR).

Retinas from the 14-day-old chick embryos were explanted to organ culture conditions in Eagle's minimal basal medium, which was supplemented with 20% dialyzed bovine fetal serum as previously described [16,17]. Sodium molybdate, to a concentration of 20 mM, was added to the culture medium, in order to inhibit the transformation of TA-GR in the intact tissue. The retinas were equilibrated in culture with $5x10^{-8}$ M [^{3}H]TA for 3h at 0-2°C. The retinas were extensively washed with ice-cold Tyrode's balanced salt solution, and rinsed with a homogenization buffer containing molybdate and PMSF. Excess of buffer was removed by 1 min centrifugation at 800g. The cytosol was prepared in TETGM-P buffer containing [^{3}H]TA, as described in section 2. Nonspecific binding of [^{3}H]TA in the cytosol of the intact tissue was determined in parallel cell-bound cytosol preparations, prepared from cultures to which 100-fold excess of unlabeled TA was added to the culture medium and to the homogenization buffer.

4) Activation of [^{3}H]TA-GR.

A) Heat-Activation. KCl (4M) was added to the [^{3}H]TA equilibrated cytosol to a final concentration of 0.15 M prior to heating. Heating at 30°C was carried out for 60 min with intermittent gentle vortexing.

B) Salt-Activation. KCl was added to the [^{3}H]TA equilibrated cytosol to a final concentration of 0.4 M. The cytosol was maintained at 0°C for additional 3h with intermittent gentle vortexing.

5) Removal of Free [^{3}H]TA, Molybdate and Salt from the Cytosol.

The cytosol was cleared of low molecular weight constituents by gel filtration (GF) on a Sephadex G-25 M column. This step was performed by one of the following procedures: a) GF on Pharmacia PD-10 prepacked columns (total volume

9ml) equilibrated in the cold with TETG buffer and eluted by gravity flow; b) Centrifugal-GF: Minicolumns (Bio-Rad) were packed with 2ml of Sephadex G-25 equilibrated with 1% synthetic carrier ampholine mixture (SCAMS), pI-range 5-8 (LKB), in 10% (v/v) glycerol. To reduce cytosol dilution, the minicolumns were partially dried by 30 second centrifugation in a MSE cell centrifuge, using a swinging bucket rotor. Cytosol aliquots of 200 μl, visualized by hemoglobin (human hemolysate) were then applied on the "dried" columns, while immersed in the ice-water bath. After a 5 min equilibration with the column matrix, the cytosol was brought to the center of the column by 200 μl SCAM solution, and then eluted by 30 second centrifugation. KCl and free [^{3}H]TA were not eluted under these conditions.

6) Electrofocusing in Cylindrical Ultrodex Gels.

A) EF in Synthetic Carrier Ampholine Mixtures (SCAMs). EF was carried out at -4°C in gel tubes of 6 or 18 mm internal diameter (i.d.) containing Ultrodex (LKB), 1% Ampholine (pI-range 5-8), in 20% (v/v) glycerol, using the apparatus and procedure described previously[1,18,19]. The sample was introduced into the gel bed under suction, followed by 1% pI-range 5-8 Ampholine in 20% glycerol. (Note that the basic SCAMs of pI-range 8-10 added to the cytosol in our previous studies[1] were here omitted). To support the Ultrodex matrix and prevent water flow, the 8 cm long gel was sealed at both ends by 15% polyacrylamide gel plugs of 1 cm length of the same buffer composition as anolyte and catholyte respectively. Catholyte was 0.1M L-lysine (colorless free base, stored at -20°C, pH 9.6, 25°C), anolyte 0.1M TES, pH 4.2 (25°C), each in 20% glycerol. EF was carried out at 25 V/cm for 18 to 20 h. The use of weakly basic catholyte and weakly acidic anolyte ensured the stability of the pH gradient for at least 30 h, at this constant voltage[18]. The gels

were then solidified in liquid nitrogen. Gel slicing (4mm slices) and pH analysis were carried out as described[19].

B) Buffer EF. EF in buffer carrier constituents was carried out as described in Section A with the following exceptions: a) gels were made in 4:1 (v/v) mixture of Poly/Sep 47 (Polysciences) and glycerol; b) catholyte was 0.1 M arginine (free-base), pH 10.5, anolyte 0.1 M lactic acid, pH 2.5, each in 20% glycerol; c) gels were pre-focused overnight at 10 V/cm prior to sample application; d) the cytosol was injected into the gel through the cathodic plug in several aliquots, using a Hamilton syringe (100 μl). A hole in the glass tube just above the anodic plug served as a pressure outlet. After injection, the hole was sealed by a rubber stopper.

7) Polyacrylamide Gel Electrophoresis (PAGE). PAGE was carried out in moving boundary electrophoresis buffer system 2964.2, using stacking gels of 5%T, 15%C (using N'N'-diallyltartardiamide as the crosslinker) in 20% glycerol at -4°C, as described previously[20,21]. The position of the moving boundary was marked by hemoglobin.

8) Protein Quantitation.

Protein was determined by the Coomassie Brilliant Blue binding as described by Bradford[22] using bovine serum albumin as the standard.

9) Isotope Analysis.

Isotope analysis was carried out on the gel slices suspended in 0.5 ml KCl (for pH analysis). The suspension of gel slices was mixed with 10 ml Scintillator 299 (Packard). Radioactivity was measured in a liquid scintillation spectrometer (Beckman No. LS 9000 or Kontron Scintillation counter Betamatic, both with a counting efficiency for tritium of 30%). Correction for counting efficiency (providing dpm) was achieved by the external standard ratio method.

RESULTS

I) EF of TA-GR formed in the cell-free cytosol.

Previous EF analyses of [^{3}H]Triamcinolone acetonide (TA)-GR complexes, formed in the cell-free cytosol of the embryonic neural retina at 0-2°C, revealed 3 major species - designated I, II and III - with apparent pI (pI') values of 5.4 ± 0.2, 6.3 ± 0.2 and 7.5 ± 0.4 respectively[1,23]. GR activation eliminated the TA-GR I fraction, slightly increased TA-GR II, and rendered TA-GR III the major component in the cell-free cytosol. Nuclear binding assays revealed that III is a nuclear binding complex[1]. Figure 1 represents a typical EF pattern of [^{3}H]TA-GR in the "nonactivated" cell-free cytosol that was exposed to basic SCAMS, as performed in our previous study[1]. To clarify the significance of the heterogeneity of GR fractions observed in EF, the possible contribution of the following factors was investigated: 1) free [^{3}H]TA, 2) basic SCAMS, 3) dilution, and 4) proteolysis. The contribution of nonspecific binding of [^{3}H]TA in the cell-free cytosol was excluded in the previous study[1].

1) TA-GR Species Multiplicity as a Function of the Presence of Free TA.

Although TA is not charged, its binding to SCAMS could form electrophoretically mobile species. To rule out this possibility, 3 types of experiments were performed: a) Isolated EF fractions I, II and III were electrophoresed on stacking gels of PAGE; b) Isolated species III was subjected to GR; and c) EF was performed on a sample of free TA.

a) Incidental to attempts at PAGE fractionation of the isolated EF components I to III[2], Ultrodex-EF gel slices corresponding in pH to the pI' of I, II and III were transplanted onto stacking gels in PAGE. The positions of the stack were excised and analyzed for bound [^{3}H]TA. The yield in case of species I and II was 70-80%, and in case of species III, 50% of the applied counts. Since free TA

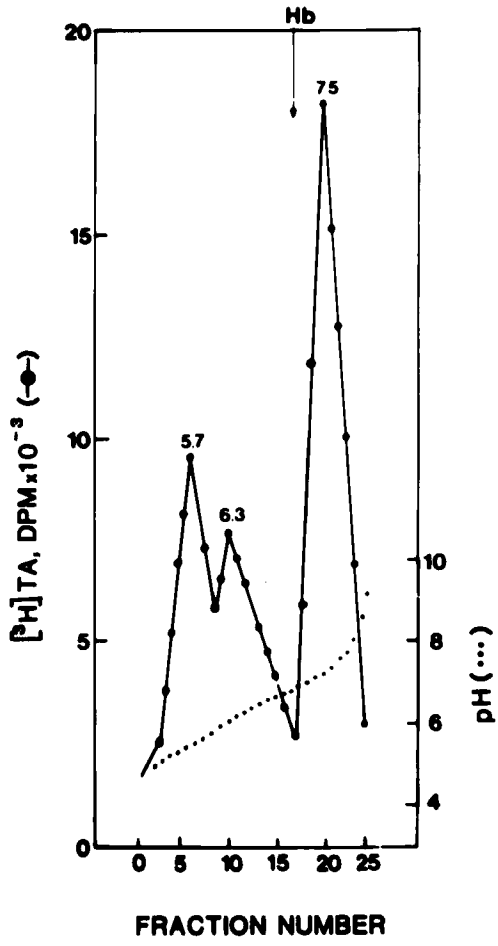

FIGURE 1

The effect of basic SCAMs on the nonactivated GR complex. The cytosol prepared in TETG buffer was equilibrated with $5x10^{-8}$M[^{3}H]TA for 2h at 0-2°C. The cytosol, visualized by hemoglobin, was cleared of free [^{3}H]TA by GF on Sephadex G-25 PD-10 prepacked columns, and was then exposed to 1% basic SCAMs, pI-range 8-10, for 5 min prior to EF. EF was carried out in 18 mm i.d. tubes containing Ultrodex in 1% SCAMs, pI-range 5-10, in 20% glycerol, at 25 V/cm for 18h at -4°C (see Methods for details of EF method). The arrow marks the site of focused hemoglobin (Hb).

does not migrate in PAGE under the conditions used, this rules out a substantial contamination of I and II by free TA.

b) TA-GR III was eluted from the Ultrodex slices and applied to a Sephadex G-25 column under the conditions described for the separation of bound from free label. More than 70% of the applied counts were eluted in the void volume.

c) The possibility that TA-GR species were formed either artifactually by EF of free TA, or by EF of TA dissociated from TA-GR under the conditions of EF, was tested. It was found that free TA, uniformly admixed to the gel, remains evenly distributed throughout the gel upon EF. When free [^{3}H]TA was loaded into the gel columns, more than 98% of the label remained at the site of application after 17-20 h of EF under the conditions used.

2) The Effects of Basic SCAMs on GR.

The considerable variability of pI' values exhibited in the three TA-GR species, (particularly apparent in TA-GR III) which seemed to vary with various basic SCAM preparations used (Table 1 of [24]), prompted us to study the effect of basic SCAMs (pI-range 8-10) on GRs. By omitting the exposure of [^{3}H]TA-GR to basic SCAMs (previously added to the cytosol in order to enhance net charge, and thus mobility), and by conducting EF in less alkaline pH gradients (using a SCAM pI-range 5-8 instead of 5-10), the experiments revealed remarkable differences in EF patterns of [^{3}H]TA-GR in the nonactivated cytosol (± molybdate) (Fig. 2A, Table I, B and C): a) TA-GR I, with pI' value 4.8, turned out to be the major species of the nonactivated state; and b) The pI' values of TA-GR II and III decreased to 5.7 and 6.2, respectively, as clearly shown in EF patterns of activated cytosols (Figs. 2B and C). The small fraction of TA-GR III found in molybdate treated cytosols exhibited a pI' value of 6.5 (Figs. 2A and D, Table I, C and F). The effects of basic SCAMs on the pI' values of the various [^{3}H]TA-GR species in the cell-free cytosol are summarized in Table I.

TABLE I: [^{3}H]TA-GR Species Revealed by EF

	Cytosol					EF					Cytosol					EF			
	Non act.	Heat act.	KCl act.	Mo	Inh.	SCAMs pI-range	Buffers	pI'	GR		Non act.	Heat act.	KCl act.	Mo	Inh.	SCAMS pI-range	Buffers	pI'	GI
A	+	-	-	-	-	5-8 8-10	- -	5.7 6.3 7.5	I II III	G	-	-	-	+	-	-	3-10[c]	4.8 5.6 8.6	I II -
B	-	-	-	-	-	5-8	-	4.8[a] 5.7 (6.2)[b]	I II III	H	-	-	-	-	-	-	3-10	(4.9) 5.7 6.5	I II III
C	+	-	-	+	-	5-8	-	4.8 5.7 (6.5)	I II III	I	+	-	-	+	-	-	3-10 + Arg.	- 5.7 5.9 8.6	- II
D	-	+	-	-	-	5-8	-	(4.8) 5.7 6.2	I II III	K	-	+	-	-	+[d]	5-8	-	(4.8) 5.4 6.2	I II III
E	-	-	+	-	-	5-8	-	4.8 5.7 6.2	I II III	L	-	+	-	-	+[e]	5-8	-	(4.8) 5.4 6.2	I II III
F	-	-	+	+	-	5-8	-	4.8 5.7 (6.5)	I II III	M	-	+	-	-	+[f]	5-8	-	(5.0) (5.4) 6.2	I II III

act: activated
Inh: protease inhibitors
Mo: molybdate

a Major species underlined
b Minor species in parentheses
c Poly/Sep 47 (Polysciences)
d In the presence of 3mM leupeptin
e In the presence of 4mM antipain
f In the presence of 1mM PMSF

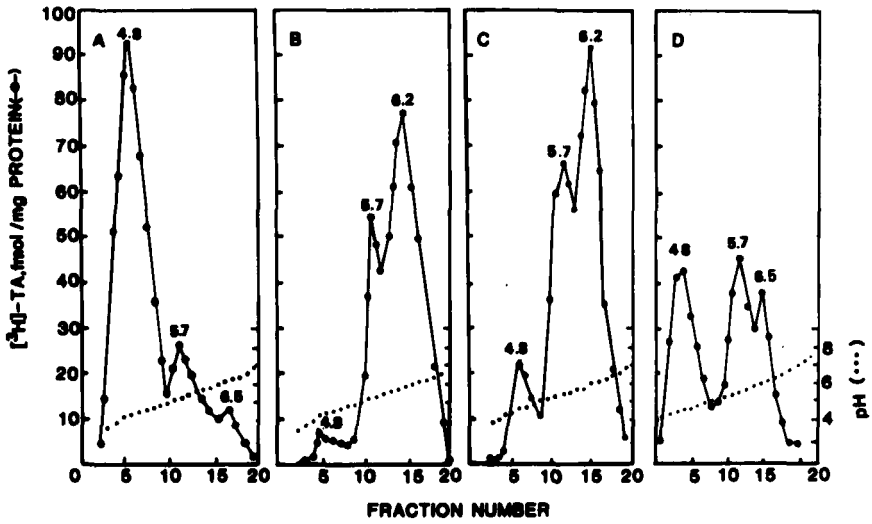

FIGURE 2

Changes in EF pattern of (^{3}H)TA-GR following activation A: Nonactivated cytosol, prepared in either TETG or TETGM buffer, and processed as described in the legend in Fig. 1, with the exception of omitting the exposure of (^{3}H)TA-GR to basic SCAMs. B: Heat-activated cytosol: Cytosol in TETG buffer equilibrated with (^{3}H)TA at 0-2°C. KCl to 0.15M was then added and the cytosol heated to 30°C for 60 min. C: Salt-activated cytosol: (^{3}H)TA equilibrated cytosol (as in B) was exposed to 0.4M KCl for 3h at 0°C. D: Molybdate-treated cytosol, exposed to 0.4M KCl: Cytosol in TETGM §20mM molybdate) equilibrated with (^{3}H)TA, and exposed to salt as in C. EF in SCAMs, pI-range 5-8, was carried out as described in the Legend to Fig. 1.

Changes in EF Pattern of [^{3}H]TA-GR Concomitant with Activation.

A) Heat-Activation. Exposure of [^{3}H]TA-GR, prepared in buffer A, to activation by heat led to the elimination of the acidic TA-GR I species, an increase in TA-GR II, (exhibiting a pI' value of 5.7) and generation of the TA-GR III, pI' value of 6.2, which represents the major GR species of the heat-activated cytosol (Fig. 2B, Table I, D).

B) Salt-Activation and the Effect of Molybdate. EF patterns of [^{3}H]TA-GR prepared in buffer A and exposed for additional 3h to 0.4M KCl, were found to be similar to those of heat-activated cytosol (Fig. 2C, Table I, E). However, quantitative differences exist. TA-GR did not disappear to the same degree as in the heat-activated cytosol, and the total binding activity (fmoles/mg protein) was better preserved in cases of salt-treatment in the cold. Treatment with 0.4M KCl of the cytosol containing molybdate modified the salt-induced transformation of TA-GR (Fig. 2D, Table I, F), causing a decrease in TA-GR I, with a corresponding increase in TA-GR II. TA-GR III was almost absent. The minor peak of TA-GR III formed exhibited a pI' value augmented from 6.2 to 6.5.

Replacement of SCAMs by Buffer Carrier Constituents.

In order to test whether the multiplicity of TA-GR species and their pI' values were generated artifactually by SCAM binding[25], the EF analysis of TA-GR was repeated using a buffer mixture (Poly/Sep 47, Polysciences) as carrier constituents. The three major TA-GR components I, II and III, were resolved in the buffer mixture as in SCAMs. The pI' values of the three TA-GR species were not significantly different from TA-GR in SCAMs. (compare Table I, sections H and D). However, a remarkable change in EF patterns of [^{3}H]TA-GR in buffer electrofocusing was observed in the analyses of nonactivated molybdate-stabilized [^{3}H]TA-GR. Three TA-GR fractions of similar size were resolved. TA-GR I with a pI' value of 4.8, TA-GR II with a pI' value 5.6 and a new basic TA-GR species with a pI' value of 8.6 (Table I, G). The nature of this "transformation" was elucidated when arginine was added to the sample phase of EF, as a control for the addition of basic SCAMS, pI-range 8-10. The exposure of molybdate-stabilized [^{3}H]TA-GR t the high concentration of arginine (0.1M, at the site of sample application) led to the elimination of the acidic TA-GR fraction (pI' 4.8) and to the appearance of most

[^{3}H]TA-GR complexes of the cytosol as a basic, pI' 8.6, species (Table I, section I). An alkaline [^{3}H]TA-GR species was not detected in buffer electrofocusing of [^{3}H]TA-GR which had not been exposed to molybdate (Table I, H), in spite of the presence of arginine in the Polyp/Sep 47 buffer mixture. As previously shown, molybdate binds to the GR complex[26]. It is therefore plausible to assume that the TA-GR species with pI' 8.6 derived from the molybdate-bound acidic species through the binding of arginine to the molybdate-TA-GR I complex.

3) The Effect of Cytosol Dilution on GR.

The application of GR on Sephadex G-25 prepacked PD-10 columns, to clear the cytosol of free [^{3}H]TA, molybdate and salt prior to EF, gave rise to a three-fold dilution of the cytosol. To assess the effects of this dilution on TA-GR, the procedure of centrifugal-GF was applied. This procedure, which diluted the cytosol only by 50-70%, almost entirely prevented the appearance of TA-GR II and III in the nonactivated cytosol. Almost all of the GRs in the nonactivated cytosol, ± molybdate, were resolved as TA-GR I, with a pI' value of 5.0 ± 0.2. [The EF patterns obtained were quite similar to those shown in Figs. 3A and D, which summarize the results of another experimental series].

4) Effect of Protease Inhibitors on GR.

To test whether the multiplicity of [^{3}H]TA-GR species revealed by EF may result from proteolytic degradation, we tested the effect of leupeptin, antipain (antiproteases that were shown to inhibit proteolysis of steroid receptors[2,15,27]) and PMSF on GR. Either leupeptin (3mM) or antipain (4mM) were included in homogenization buffer A and the cytosols were subjected to heat-activation. These treatments did not change the multiplicity of [^{3}H]TA-GR species in EF, but did cause a slight shift in pI' of TA-GR II from 5.7 to 5.4 (Table I, K and L). In the presence of PMSF, almost all the [^{3}H]TA-GR complexes of the nonactivated cytosol,

prepared in TETG or TETGM, were in the form of TA-GR I, pI' value 5.0-5.2 (Fig. 3A and D respectively). The transformation of TA-GR I to the more basic pI' species (in cytosol preparations without molybdate) by salt or by heat was not significantly affected by the presence of PMSF (Figs. 3B and C respectively). Both species TA-GR II and III were formed; TA-GR II as a minor component (not always well resolved) and III as the major GR species in the activated cytosols. In the presence of PMSF, as in the presence of leupeptin and antipain, TA-GR II exhibited a pI' value of 5.4, (Table I, M) instead that of pI' 5.7 observed in the absence of antiproteases.

Figure 3E depicts the EF pattern of salt-treated cytosol containing PMSF in the presence of molybdate. As shown, molybdate completely prevented the formation of III induced by salt. TA-GR II appeared as a minor fraction. The comparison between Figs. 3E and 2D reveals that the generation of TA-GR II by salt in the molybdate-treated cytosol was greatly reduced (Fig. 3E). However, the decrease in the rate of transformation of TA-GR I to II was found to be correlated with cytosol dilution and not with the presence or absence of PMSF.

Figure 3F presents the transformation of TA-GR I by heat in the presence of molybdate and PMSF. The EF pattern obtained suggests that GR-transformation takes place under these conditions, but at a significantly slower rate than in the absence of molybdate. This implies that the inhibitory effect of molybdate is reversible at 30°C.

II) EF of Cell-Bound TA-GR.

In order to validate the biological significance of TA-GR I detected in the nonactivated cell-free cytosol, [^{3}H]TA-GR formed in the cytosol of the intact tissue in organ culture at 0-2°C was also analyzed by EF. Molybdate was added to the medium to a concentration of 20mM in order to inhibit transformation of TA-GR

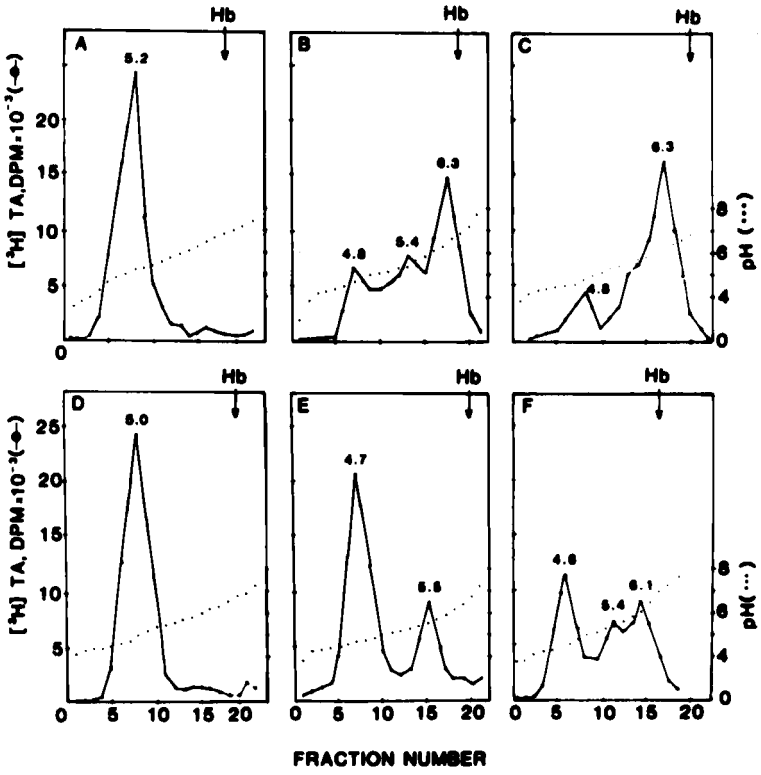

FIGURE 3

Effects of molybdate, cytosol dilution and PMSF on EF patterns of [^{3}H]TA-GR in nonactivated and activated cytosols. The cytosol prepared in buffer C (TETG-P) or buffer D (TETGM-P), was divided into 3 equal portions. One portion remained nonactivated and was maintained at 0°C, one was treated with 0.4 M KCl at 0°C and one was activated by heat. The cytosols were cleared of free [^{3}H]TA, molybdate and salt by centrifugal-GF in minicolumns of Sephadex G-25 equilibrated in 1% SCAMs, pI-range 5-8 (see Methods for details), thereby reducing the extent of cytosol dilution in GF. A and D, nonactivated cytosols. B and E, salt-activated cytosols. C and F, heat-activated cytosols. EF was carried out in 6mm i.d. tubes containing Ultrodex, in 1% SCAMs, pI-range 5-8, at 25 V/cm for 17h at -4°C (see Methods for details).

that might occur in the intact tissue. The retinas were exposed to [^{3}H]TA (± 100-fold of unlabeled TA) after 30 min in culture with molybdate. Saturation of bound [^{3}H]TA-GR was achieved after 3 to 4 h. There was no specific binding of [^{3}H]TA-GR to the nuclei under these conditions. The cytosol prepared in TETGM buffer containing 1mM PMSF and [^{3}H]TA (± unlabeled TA) was cleared of the low molecular weight constituents by GF on a Sephadex G-25 PD-10 column. Fig. 4 shows that the EF pattern of nonactivated cell-bound [^{3}H]TA-GR, stabilized in the intact cells by molybdate, is similar to the EF pattern of 'nonactivated' TA-GR formed in cell-free cytosol which was diluted to the same extent (Fig. 2A). Most of the [^{3}H]TA-GR complexes were resolved as TA-GR I. The formation of small fractions of TA-GR II and III in the cell-bound preparation was prevented when the dilution factor was reduced by centrifugal-GF (data not shown). However, the pI' value of cell-bound TA-GR I in nonactivated cytosol was consistently found to be lower (4.2 ± 0.2) than that exhibited in the cell-free cytosol (5.0 ± 0.2). Nonspecific binding of [^{3}H]TA in the cytosol of the intact cells is negligible (Fig. 4), as previously shown for cell-free preparations[1].

DISCUSSION

The monitoring by EF of changes in the charge properties of TA-GR concomitant with transformation of the GRs in the cytosol of the neural retina was impeded by two main problems, the high sensitivity of the nontransformed [^{3}H]TA-GR complex to temperature, and the transforming effects of basic synthetic carrier ampholyte mixtures (SCAMS) on the GR complex. To solve the first problem, Joule heating in EF was reduced by: a) establishing the experimental conditions for attaining the steady-state condition in EF at very low initial currents[18]; and b)

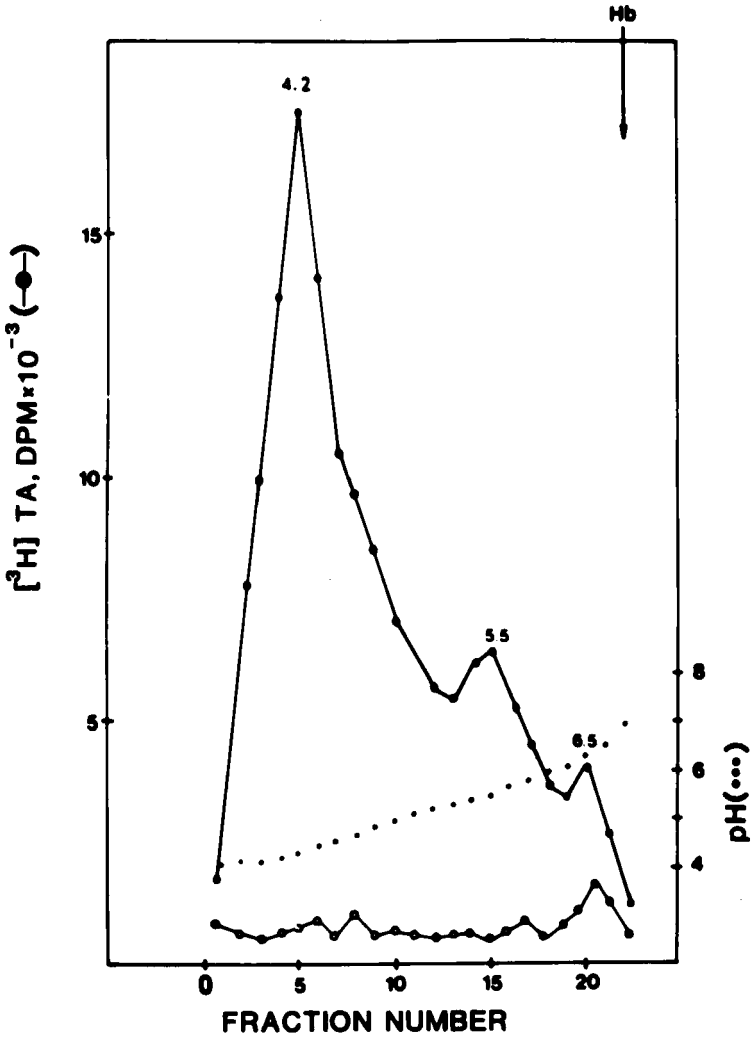

FIGURE 4

EF pattern of nonactivated cell-bound [^{3}H]TA-GR. Neural retina from 14-day-old chick embryos were equilibrated with $5x10^{-8}$M [^{3}H]TA under organ culture conditions in medium containing 20mM molybdate, for 3h at 0°C. The tissue was homogenized in TETGM-P buffer containing free [^{3}H]TA. The cytosol was cleared of free [^{3}H]TA and molybdate by GF on a Sephadex G-25 PD-10 column. EF was carried out in 6mm i.d. tubes in Ultrodex, 1% SCAMs, pI-range 5-8. The nonspecifically bound [^{3}H]TA in the cytosol (o----o), was determined in parallel cell-bound preparations in which 100-fold excess of unlabeled TA was added to the culture medium and to the homogenization buffer.

developing the method of EF in tubes containing Ultrodex, which allows efficient control of the sample temperature in EF at -4°C[20]. EF of [^{3}H]TA-GR at such strictly controlled cold temperature allowed for the detection of the acidic, temperature sensitive TA-GR I species in the nonactivated cytosol[1]. The second problem was solved by omitting pI 8-10 SCAMs from the system. This omission prevented the uncontrolled transformation of TA-GR I to the activated species. It also reduced the pI' values of the TA-GRs. The artifactually increased pI' values of GR species in the presence of basic SCAMs are probably due to their binding to GR[28]. The binding of basic SCAMs to TA-GR may cause the dissociation of a negatively charged low molecular weight inhibitor which has been postulated to explain GR activation by dilution and gel filtration[30] (see below). These actions of basic SCAMs on GR are apparently similar to the activating effect of alkaline pH[29] and/or basic amino acids and other primary amines[30] on GR. The agreement between the pI' values of GR derived from pH-gradients established by SCAMs, pI-range 5-8, and those obtained in pH-gradients established by simple buffers (Poly Sep/47) (Table I, H) suggests that the pI' values of GR reported in this study reflect the authentic net charge of GR.

The omission of basic SCAMs in this study remarkably reduced the heterogeneity of GR in the nonactivated cytosol. However, minor fractions of TA-GR II, and to a lesser extent III, were still found in the 'nonactivated' cytosols (Figs. 2A and 4). This partial transformation appears to have been induced by dilution of the cytosol during GF on Sephadex G-25 (PD-10 column). This partial transformation was successfully controlled by decreasing the extent of cytosol dilution prior to EF (Figs. 3A and D).

These experiments clearly demonstrate the charge homogeneity of the GRs in the nonactivated cytosol and the acidic nature of the nonactivated GR complex.

These conclusions, derived from studies on the cell-free cytosol, are supported by imilar results obtained by EF analyses of nonactivated, cell-bound [^{3}H]TA-GR (Fig. 4), and the nonactivated partially purified 350 kDa [^{3}H]TA-GR complexes, (Fig. 6A in [2]). However, as presented in Fig. 4, the pI' value of nonactivated cell-bound TA-GR I is more acidic (pI' 4.2 ± 0.1) than the pI' values observed in cell-free preparations (pI 5.0 ± 0.2). The increase in pI' value of TA-GR I from 4.2 ± 0.1 to 5.0 ± 0.2 may result from the removal of the proposed negatively charged inhibitor of GR transformation from the GR complex by the dilution of the holoreceptor in the cell-free cytosol with homogenization buffer[30]. It was shown that binding of the hormone to the receptor stabilizes the GR complex[31-33]. It appears that the binding of the hormone to GR in situ (in the cold) stabilizes the bond between TA-GR I and the putative negatively charged inhibitor, thereby conferring resistance to its loss by dilution in the cell-free preparation. This suggestion is supported by observations reported in other glucocorticoid target tissues (Reviewed in [9, 34]; see also [35]) on the involvement of a negatively charged low molecular weight constituent in the stabilization of the GR complex in the nonactivated state. The dissociation of this putative negatively charged constituent from the nonactivated [^{3}H]TA-GR complex may reflect the primary event in GR transformation.

Acidic GR species were also detected in other GC-target cells. GR with a pI' value of 4.0 was observed in EF of cell-bound mouse L-cells[12]. GR with a pI' value of 5.2 was detected in cell-free cytosols of IM-9 human lymphoid cells[12]. It is plausible to assume that these GR forms are analogous to TA-GR I in the cytosol of the neural retina. The observed heterogeneity of GRs in the nonactivated state, and the similarity in the distribution of the various GR charge-species between the nonactivated and the activated cytosols in these studies, have to date obscured the significance of the most acidic GR form.

Activation by heat or by salt caused the transformation of TA-GR I to two more basic forms: TA-GR II with pI' 5.6 ± 0.2 and TA-GR III with pI' 6.2 ± 0.1. TA-GR II was always detected, as a minor component, and TA-GR III as the major GR form in the fully activated cytosol. These two pI' species were also resolved by EF of partially purified 4S [^{3}H]TA-GR, fractionated from heat or salt-activated cytosols (Fig. 6C in [2]). The nature of TA-GR of pI' 5.6 in the activated cytosols is not clear. It was shown in other GR systems that a GR species with a pI' value of 5.9 may represent a proteolytic product of the pI' 6.1-6.3 species[14,15]. Other investigators suggested that GR species with pI' values of 5.4 and 6.1-6.3 may be products of different genes[36]. It appears unlikely that TA-GR with pI' 5.6 in the activated cytosol represents a proteolytic product since size determinations of GR, in parallel [^{3}H]TA-GR preparations, have shown that the 90 kDa [^{3}H]TA-GR is the smallest GR complex detected in the activated preparations[2]. The heterogeneity in charge properties of the activated GR species may, however, relate to the recent finding that the GR transcript contains several internal initiation sites that yield multiple protein species[37].

Molybdate ions prevent the formation of III. The inhibitory effect of molybdate on GR transformation is apparently reversible at 30°C (Fig. 3F). The effect of molybdate on the GR complex is, however, expressed in the salt-treated preparation. The EF feature that characterizes the salt-treated molybdate-stabilized GR (in addition to the absence of III) is the appearance of a well resolved TA-GR II, pI' 5.6, fraction. The relative amount of [^{3}H]TA-GR that is resolved as II (in relation to [^{3}H]TA-GR I and to the total-bound [^{3}H]TA-GR in the cytosol) appears to relate to the dilution of the preparation during its clearance of salt, using filtration on Sephadex G-25, just prior to EF. The ratio II/I increases with the increase in [^{3}H]TA-GR dilution (compare Fig. 2D to Fig. 3E). A plausible solution

to this puzzle is suggested by the recent results which were obtained from EF analysis of the partially purified [^{3}H]TA-GR, fractionated from the molybdate-stabilized salt-treated cytosol as a 170 kDa complex (S=5.1; R_S=7.8nm). All the [^{3}H]TA-GR complexes that were present in the sample were resolved in a homogeneous sharp peak with a pI' value of 5.6±0.2 (i.e. a II). These observations support the suggestion that II represents an intermediate state in GR transformation. These observations also suggest that the appearance of [^{3}H]TA-GR I (pI' 5.0) in EF of the parallel crude cytosol preparation may result from the reassembly of II to I when cleared of salt just prior to EF. The apparent reassembling of the 170 kDa complex (TA-GR II) to TA-GR I (350 kDa complex) is feasible in the crude cytosol preparations (Figs. 2D, 3E) but <u>not</u> in the partially purified 5.1 S fraction (Fig. 6B in [2]). The apparent reassembly of I from II in the crude cytosol is concentration-dependent; it is more pronounced in the more concentrated preparation (Fig. 3E).

It appears that [^{3}H]TA-GR complexes with a pI' value of 5.6 may represent two different size forms; one corresponds to the 170 kDa complex which was fractionated from the salt-treated molybdate-stabilized preparation; the second corresponds to the 90 kDa form which was fractionated as a minor constituent from heat- or salt-activated preparations. However, the possibility that the latter may correspond to a larger GR complex, formed from the isolated 90 kDa complex (pI' 6.2) under the low salt conditions of EF, cannot be excluded.

The study of the charge properties of the GRs of the neural retina by EF under controlled nondenaturing conditions suggests that GR transformation is a multistage process, characterized by a gradual decrease in the negative net charge of the hormone-receptor complex. The nature of these transformations will be further discussed in a separate study in correlation with the changes in the molecular weight of the GR complex[2].

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