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CHARACTERIZATION OF CYTOSOLIC GLUCOCORTICOID RECEPTOR OF FETAL RAT EPIPHYSEAL CHONDROCYTES

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Summary—The cytosolic glucocorticoid receptor of 21st gestational day rat epiphyseal chondrocytes has been evaluated. The receptor, a single class of glucocorticoid binding component approached saturation, utilizing [^{3}H]triamcinolone acetonide ([^{3}H]TA) as the radiolabeled ligand, at approximately 1.8–2.0 $\times 10^{-8}$ M. The dissociation constant (K_d) reflected high-affinity binding, equaling 4.0 $\pm$ 1.43 $\times 10^{-9}$ M ($n = 7$) for [^{3}H]TA. The concentration of receptor estimated from Scatchard analysis was approximately 250 fmol/mg cytosolic protein and when calculated on a sites/cell basis equalled 5800 sites/cell. The relative binding affinities of steroid for receptor were found to be triamcinolone acetonide > corticosterone > hydrocortisone > progesterone > medroxyprogesterone acetate $\gg$ 17α-hydroxyprogesterone $\gg$ testosterone > 17β-estradiol. Cytosolic preparations activated *in vitro* by warming (25°C for 20 min) were shown to exhibit an increased affinity for DNA–cellulose. 46% of the total specifically bound activated ligand–receptor complex was bound to DNA–cellulose. Cytosol maintained at 0–4°C in the presence of 10 mM molybdate or activated *in vitro* in the presence of molybdate, bound to DNA–cellulose at 8 and 10% respectively. DEAE–Sephadex elution profiles of the nonactivated receptor were indicative of a single binding moiety which eluted from the columns at 0.4 M KCl. Elution profiles of activated receptor were suggestive of an activation induced receptor lability. The 0.4 M KCl peak was diminished, while a concomitant increase in the 0.2 M KCl peak was only modestly discernible. Evaluation of endogenous proteolytic activity in chrondrocyte cytosol using [methyl-^{14}C]casein as substrate show a temperature-dependent proteolytic activity with a pH optimum of 5.9–6.65. The proteolytic activity was susceptible to heat inactivation and was inhibitable, by 20 mM EDTA. The sedimentation coefficient of the nonactivated receptor was 9.3*s* ($n = 6$) on sucrose density gradients and exhibited steroid specificity and a resistance to activation induced molecular alterations when incubated in the presence of 10 mM molybdate. Receptor activation *in vitro*, in the absence of molybdate induced an increased receptor susceptibility to proteolytic attack and/or enhanced ligand receptor dissociation as evidenced by a diminution of the 9.3*s* binding form without a concomitant increase in 5*s* or 3*s* receptor fragments.

INTRODUCTION

The focus of endochrondral growth in the developing long bone is the growth plate and the influence of glucocorticoids on the chondrocyte components of this cartilagenous tissue is of critical importance. Growth reductions in glucocorticoid-affected endochondral bone have been attributed to premature maturation characterized by precocious calcification; the induced precocious calcification has been clearly demonstrated *in vivo* [1, 2–4]. Glucocorticoid-induced narrowing of the epiphyseal growth plate has been demonstrated [5, 6] and has been attributed to developmental arrest of the chondrocytes. Chondrocyte number and size in the proliferative and hypertrophic zones of the epiphyses of adult rats [7] and condylar cartilage of neonatal mice [8] have been shown to be reduced following corticosteroid exposure. Suppression of osteogenic and chondrogenic cell proliferation by corticosteroids has also been noted [9, 10]. The suppression of cell proliferation has been shown to be corticosteroid dose-dependent and related directly to increased levels of RNA and protein synthesis [11, 12].

High-affinity, specific glucocorticoid receptors have been identified in neonatal mandibular condyles [13], in whole cell cultures of rabbit articular condyles [14], in dog epiphyseal chondrocyte cell suspensions [15], and in cytosol prepared from the epiphyses of fetal chick long bone [16]. Such studies

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have utilized primarily binding kinetics and specificity analyses. To evaluate the physiological mechanisms of glucocorticoid action in cartilagenous tissue, an evaluation of post ligand–receptor binding events is needed. These post ligand–receptor binding events include the process of receptor activation and subsequent increased affinity of activated receptor for DNA as well as modulated transcriptional activity and RNA stability.

The ontogeny and role of steroid receptors in the developing embryo is of particular interest. The present studies have been undertaken to characterize the cytosolic glucocorticoid receptor of fetal rat epiphyseal chondrocytes in order to establish base line biochemical parameters of the hormone receptor extending beyond classical binding kinetics analyses. It is anticipated that these fundamental functional characteristics will provide a basis with which to understand endochondral bone growth retardation which may be a resultant of glucocorticoid receptor dysfunction.

We have characterized the chondrocyte glucocorticoid receptor utilizing saturation and Scatchard analyses, binding specificity analysis and have evaluated the functional capacity of the receptor to undergo activation. The receptor has been characterized by assessing *in vitro* activation-induced alterations in anion exchange elution profiles, increases in DNA binding capacity, and changes in sucrose density gradient sedimentation coefficients.

METHODOLOGY

All laboratory animals utilized in these studies were maintained in strict accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23).

Adult (175–225 g) female Wistar rats (Marland Farms, N.J.) were utilized. The required timed pregnant animals were obtained by placing individual nulligravid females with males overnight. Each mated animal was evaluated between 0900 and 1000 the following morning. The presence of sperm in the vaginal lavage was indicative of the first day of gestation, the embryo was designated to be zero days old.

Triamcinolone acetonide (TA), (9-α-fluor-11, β21-dihydroxy-16,17[(1-methyl-ethylidene)bis-(oxy)] pregna-1,4-diene-3,20 dione) 1,24[^{3}H], 20–30 Ci/mM was used as the ligand to measure the glucocorticoid receptor [17]. The above and nonlabeled Org 2058 were purchased from Amersham. Nonlabeled TA and other radioinert steroids, calf thymus DNA and Tris were obtained from Sigma. DEAE–Sephadex A-50, Sephadex G-25 PD10 columns and Dextran T500 were from Pharmacia; hydroxylapatite was from BioRad. Other chemicals including scintillation counting fluid (Scintiverse 1) were from Fisher Scientific. A Beckman Scintillation Counter was used for isotope counting using appropriate internal standards to determine disintegrations per min (DPM).

Chondrocyte isolation from fetal endochondral bone

Pregnant rats were killed via cervical dislocation on the 21st gestational day. Utcrotomies were performed and the fetuses were dissected free of their supporting membranes, decapitated and the limbs extirpated and pooled in ice-cold phosphate buffered saline (PBS). After careful removal of any connective or muscle tissue, the fetal limbs were enzymatically cleared of any adhering tissue by incubation in a solution consisting of 0.1% collagenase (Worthington Type II) in calcium, magnesium-free PBS (pH 7.4) at 21°C for 10 min [18]. To ensure minimal cell damage induced by clostripain and various cytotoxic enzymes within bacterial collagenase, all tissue preparations were preincubated in the presence of α-tosyl-L-lysyl-chloromethylketone (TLCK, 7 nM/mg collagenase (from Sigma) prior to enzymatic digestion [19]. Following this clearing incubation, the epiphyses were separated via blunt dissection from the diaphyses at the chondroosseous junction and were pooled in ice-cold PBS. The epiphyseal tissue was prepared for cell extraction by fine mincing.

Chondrocytes were isolated from the cartilagenous tissue utilizing a digestion medium consisting of 0.2% collagenase Type II in a magnesium free cell support buffer containing; 25 mM Hepes, 10 mM $NaHCO_3$, 110 mM NaCl, 3 mM K_2HPO_4, 24 mM KCl, 1.3 mM $CaCl_2$ 11 mM glucose, 2 mg/ml bovine serum albumin (fraction V, Sigma) pH 7.4 [20]. After gassing with N_2:O_2:CO_2 (75:20:5%) the tissue was incubated in plastic 125 ml Erlenmeyer flasks in a shaking water bath, 90 oscillations/min at 37°C for 4 h. In some instances chondrocytes were isolated by sequential digestion where suspended cells were collected at hourly intervals according to the procedures outlined below. The washed cells were maintained on ice and the enzyme solution was returned to the digestion vessel. These latter procedures allowed for increased digestion periods resulting in increased cell yield and viability.

Following incubation, the medium was decanted through several layers of cheesecloth and a 35 μ nylon mesh filter into plastic conical centrifuge tubes. The remaining tissue was washed several times with collagenase free cell support buffer which was subsequently added to the appropriate tubes. The cell suspensions were pelleted by centrifugation at 800 *g* for 10 min at 0–4°C and washed three times with cold collagenase free cell support buffer. The cell pellet was resuspended in an appropriate volume of ice-cold TESH buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), pH 7.4) in the presence or absence of 10 mM molybdate. Cell density and viability were determined utilizing trypan blue exclusion and counting with a Neubauer Chamber.

Cytosol preparation

Following resuspension in TESH buffer the chondrocytes, maintained at 0–4°C, were disrupted using one 10-s burst/min × 5 min of a Branson probe sonicator. Cytosol was prepared by centrifugation of the sonicate at 85,000 *g* for 1 h. The supernatant was decanted and used fresh. The protein content of cytosols ranged from 1.0 to 3.0 mg of protein per ml. Protein determinations were according to the method of Lowry *et al.*[21] as modified for sulfhydryl containing compounds [22].

The analyses of binding kinetics for the glucocorticoid receptor were patterned after the methods of steroid receptor analysis developed by Korenman *et al.*[23] as modified by Mester *et al.*[24]. The determination of dissociation constants (K_d) were according to the method of Scatchard[25]. For measurement of binding sites, K_d and binding time-course and temperature assays, aliquots of prepared cytosols were incubated with appropriate concentrations (1.0–20.0 nM) of [^{3}H]TA in the absence or presence of a 100-fold molar excess of radioinert TA. Following incubation (4–18 h) at 4°C (or the appropriate temperature for binding time-course assays) an equal volume of a 60% slurry of hydroxylapatite (HAP) in TESH buffer was added. The HAP cytosol slurry was incubated further for 1 h at 4°C with intermittent gentle vortexing. 2 ml of TE buffer (10 nM Tris, 1 nM EDTA, pH 7.8) were subsequently added and the HAP suspension was pelleted via centrifugation at 2500 *g* for 10 min. The pellet was washed with three 2 ml portions of cold TE buffer and finally resuspended in 1.0 ml of absolute ethanol. After incubation at room temperature for 1 h, the HAP–ethanol suspension was pelleted and an aliquot of the supernatant was removed for scintillation counting. Specific binding was calculated by subtraction of the radioactivity in the presence of excess unlabeled ligand from that measured in its absence.

Steroid specificity analysis

In order to evaluate the relative effectiveness of competitive steroid binding to receptor, aliquots of prepared cytosol were incubated with solutions containing 20 nM labelled [^{3}H]TA plus or minus 1-, 10-, 100-, 1000-, or 10,000-fold concentrations of competing steroid. The concentration of ethanol used for solubilizing competing ligands and added to the reaction mixture was shown to exert no significant effect on binding in this system. Displacement of [^{3}H]TA from the glucocorticoid receptor by competitive ligands was evaluated by plotting the percentage of [^{3}H]TA bound versus the fold concentration of competing steroid.

Anion exchange chromatography

Procedures for the chromatographic analysis of the activated and nonactivated forms of glucocorticoid receptor were adapted from those previously described [26, 27]. Columns of DEAE–Sephadex A-50 in 10 ml plastic syringes were poured using a 1:1 slurry of DEAE–Sephadex and a starting buffer consisting of 5 mM $KHPO_4$, 1.0 mM dithiothreitol (DTT), and 1.5 mM EDTA with or without 10.0 mM Na_2MoO_4, pH 7.3. The DEAE–Sephadex slurry was layered above a 2.0 ml cushion of slurry containing dextran coated charcoal. Linear gradients of 20 mM potassium phosphate buffer, 1.0 mm DTT, pH 6.8, containing 0–1.0 M potassium chloride, were used to elute the receptors.

1.0 ml aliquots of cytosol were incubated for 18 h at 0–4°C with 50 nM [^{3}H]TA in the presence or absence of 10 mM Na_2MoO_4. Molybdate containing samples were applied directly to DEAE–Sephadex columns; those not containing molybdate were activated *in vitro* by incubation at 25°C for 20 min and were subsequently applied to the column. Eighty 0.8 ml gradient fractions were collected directly into 7.0 ml polypropylene scintillation vials. Alternate fractions were used for conductivity measurements (utilizing a Markson Model No. 10 conductivity meter) or to determine HAP or DNA–cellulose binding; to others, 5.0 ml of scintillant was added in order to assess radioactive ligand concentrations.

In some chromatographic experiments a protease inhibitor cocktail was added to cytosol preparations prior to *in vitro* activation. The inhibitors and their final concentrations per ml of cytosol were pepstatin A (10 μg), leupeptin (50 μg), bacitracin (100 μg), and aprotonin (50 μg) [28].

DNA-cellulose binding

Aliquots of cytosol were incubated in the presence of 20 nM [^{3}H]TA with or without 10 mM molybdate for 18 h at 0–4°C. The cytosol prepared in this manner was maintained at 0–4°C under nonactivating conditions or subsequently activated *in vitro* by warming at 25°C for 20 min.

A pellet of DNA–cellulose was obtained from centrifugation of a 0.2 ml aliquot of a 25% slurry of DNA–cellulose prepared according to the methods of Alberts and Herrick[29]. To this pellet, a 0.1 ml aliquot of the prepared cytosol was added. The cytosol DNA–cellulose slurry was incubated for 1 h at 0–4°C. The DNA–cellulose cytosol slurry was resuspended at regular intervals via gentle vortexing.

Following the appropriate incubation period, 2.0 ml of ice cold TE buffer was added to each sample. The suspension was centrifuged at 2500 *g* in a refrigerated centrifuge for 10 min. The pellet was washed three times with 2.0 ml portions of ice-cold TE buffer and finally resuspended in 0.8 ml of TE buffer of which 0.5 ml aliquots of the resulting uniform suspension were removed for scintillation counting.

Protease assay

The characterization of protease activity in cytosol prepared from fetal rat chondrocytes was according to the methods of Mayer *et al.*[30]. Proteolytic

activity was assessed by the release of ^{14}C-labeled peptide fragments into the trichloroacetic acid (TCA) soluble fraction during degradation of [methyl-^{14}C]casein (0.5–5.0 μCi/mg, New England Nuclear). Duplicate 30 μl aliquots of cytosol were added to 1.5 ml Eppendorf tubes. The cytosol and all reaction components were preincubated at the appropriate temperature in order to insure uniform temperature-dependent reaction kinetics. The reaction was initiated with the addition of 25 μl of the casein substrate mixture containing 0.2 μCi/ml of [methyl-^{14}C]casein and 1 mg/ml unlabeled carrier casein in 40 mM potassium phosphate buffer, pH 7.6. Following incubation for 20, 40 and 60 min at the appropriate temperature, the reaction was terminated by the addition of 50 μl of 10% TCA. Insoluble material was pelleted by rapid centrifugation and 50 μl aliquots of the supernatant were removed and counted. Control reaction mixtures included blanks where buffer was substituted for cytosol and where cytosol proteolytic activity was inactivated by boiling. In experiments where pH-dependent reaction kinetics were evaluated, cytosol was prepared in TESH buffer with the pH adjusted to 5.9, 6.65 or 7.4. pH levels above 7.4 precipitated proteinaceous components of the reaction mixture and thus, these analyses were precluded.

Sucrose gradient ultracentrifugation

For density gradient ultracentrifugation of the glucocorticoid receptor [31], aliquots of cytosol were incubated at 0–4°C for 18–24 h with 50 nM of [^{3}H]TA in the presence or absence of 100-fold concentrations of cold competing steroid. Additionally, some samples were treated with 10 mM molybdate. Following incubation, 0.1 ml aliquots of the cytosol were layered on linear 5–20% sucrose gradients in a buffer consisting of 0.01 M Tris–HCl, 1.0 mM EDTA, 0.01 M thioglycerol and 0.01 M KCl, pH 7.5. Alternatively, samples were subjected to activation *in vitro* by warming (25°C for 20 min) prior to application to the sucrose gradients. The gradients were then centrifuged in a swinging bucket rotor for 16 h at 149,000 *g*. Crystalline bovine serum albumin (BSA, 4.4 *s*) and aldolase (7.9 *s*) were also layered on separate gradients and utilized as standards for determination of *s*-values. Following centrifugation, the bottom of each gradient tube was pierced and 10 or 20 drop fractions were collected directly into scintillation vials. Radioactivity or absorbence at 280 mμ were then evaluated.

Statistical analysis

Where applicable, data are presented as the mean $\pm$ SD. These data were calculated utilizing the Systat® software package (Systat Inc.). For analysis of DEAE-Sephadex elution profiles, the CUE-2 image analysis system (Olympus Corp.) was utilized to assess the area under profile peaks.

RESULTS

Chrondrocyte isolation

Collagenase digests of 1–2 g of fetal epiphyseal tissue isolated from 21st gestational day rat endochondral bone generally yielded $3.9 \pm 1.18 \times 10^7$ cells/ml ($n = 8$). The percent viability of these isolated chondrocytes was 78 $\pm$ 10% ($n = 6$). A high degree of cellular homogeneity, based on the morphological characteristics of size and shape were apparent when cellular suspensions were evaluated microscopically. Alcian blue stained tissue sections from fetal epiphyses exhibited homogeneous metachromicity and cellular homogeneity characterized by small circular chondrocytes embedded in abundant extracellular matrix. The morphological characteristics of these tissue sections were highly analogous to resting/reserve zone cartilage. The preparations were free of contaminating tissue. In this regard, Daughaday and Mariz[32] note that cartilageneous tissue is uniquely suited for biochemical analysis in that it is composed predominantly of one cell type.

Binding time-course analysis

Binding time-course and temperature analysis (Fig. 1) indicate that the specific receptor binding as assessed by HAP assay approached saturation at 4 h when the cytosolic preparation was maintained at 0–4°C. Subsequent binding at 0–4°C however, increased only modestly. In light of these data, further binding analyses were allowed to equilibrate at 0–4°C from 4 to 18 h. Analysis of binding activity at 23°C revealed that [^{3}H]TA bound to the glucocorticoid receptor maximally at 4 h. Binding subsequently declined reaching a nadir at 16 h. The reduction in binding at 16 h was approximately 45% below maximal binding activity observed at 4 h.

Saturation and Scatchard analysis

A representative saturation analysis, indicative of a high degree of specific binding of TA to the cytosolic

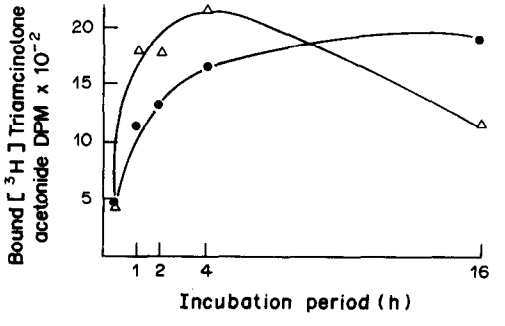

Fig. 1. Time-course of specific [^{3}H]triamcinolone acetonide (TA) binding to fetal rat epiphyseal chondrocyte cytosolic protein. [^{3}H]TA (2.0×10^{-8} M) was incubated in the presence or absence of a 100-fold molar excess of radioinert competitive ligand at 0–4°C (●) or 20–23°C (△) with the 85,000 *g* fraction of chondrocyte cytosol. Bound steroid was separated from free using hydroxylapatite (HAP).

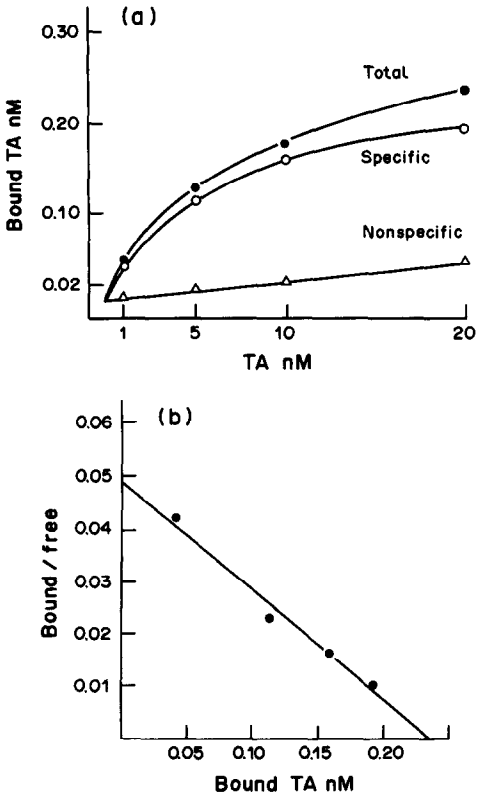

Fig. 2. [^{3}H]TA (1.0×10^{-9}–2.0×10^{-8} M) was incubated in the presence or absence of a 100-fold molar excess of radioinert competitive ligand at 0–4°C for 18–24 h with the 85,000 *g* supernatant fraction of chondrocyte cytosol. Incubation mixtures included 10 mM molybdate. (a) Saturation analysis of [^{3}H]TA binding to fetal rat epiphyseal chondrocyte cytosolic protein. The representative binding curve illustrates total (●), nonspecific (△), and specific (○) binding of [^{3}H]TA. Bound steroid was separated from free using HAP. (b) Scatchard analysis of [^{3}H]TA binding to fetal rat epiphyseal chrondrocyte cytosolic protein. Bound steroid was separated from free using HAP. Data reflecting bound/free ratios vs the amount of [^{3}H]TA bound in nM concentrations were linearly regressed. The slope of the regression line equalled the dissociation constant ($K_d = 4.8 \times 10^{-9}$ M) with a correlation coefficient ($r = 0.99$) and where the x-intercept reflects the number of high-affinity binding sites (n or $B_{max} = 234$ fmols).

receptor is illustrated in Fig. 2a. The data also show that receptor saturability was approached at 1.8–2.0×10^{-8} M. The straight line nature (correlation coefficient = 0.99) of the representative Scatchard analysis depicted in Fig. 2b, indicates the presence of a single class of glucocorticoid binding component. The affinity constant (K_d) was $4.0 \pm 1.43 \times 10^{-9}$ M ($n = 7$). The concentration of receptor estimated from Scatchard analysis was 246 (range 300–193) fmol/mg cytosolic protein. The approximate number of receptor sites per cell was calculated to be 5750 ± 2180 ($n = 5$) and is in all likelihood an underestimation due to receptor occupancy by endogenous steroid. The data concerning receptor sites/cell are in close agreement with that

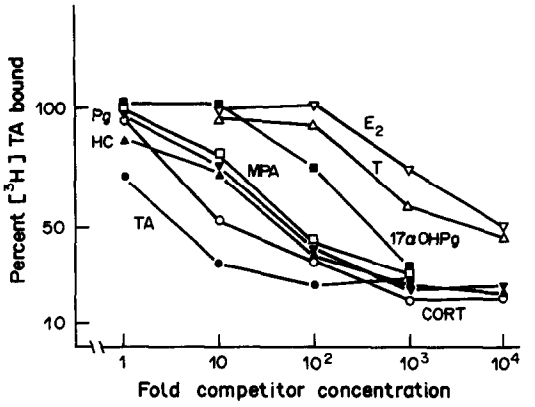

Fig. 3. Specificity of [^{3}H]TA binding to fetal rat epiphyseal chondrocyte cytosolic protein. [^{3}H]TA (2.0×10^{-8} M) was incubated at 0–4°C for 18–24 h in the presence or absence of 1-, 10-, 100-, 1000- or 10,000-fold molar concentration excesses of the indicated radioinert competitive ligand with the 85,000 *g* supernatant fraction of chondrocyte cytosol. Incubation mixtures contained 10 mM molybdate. Bound steroid was separated from free using HAP. Data are presented as the percentage of [^{3}H]TA bound to cytosolic protein. Triamcinolone acetonide (TA), corticosterone (CORT), hydrocortisone (HC), progesterone (Pg), medroxyprogesterone acetate (MPA), 17 hydroxyprogesterone (17α-OH Pg), 17β-estradiol (E_2), testosterone (T).

presented previously by Feldman *et al.*[33] where rat calvarial osteoblasts were shown to contain approximately 6000 sites/cell. Similarly, Kan *et al.*[15] have noted that chondrocytes isolated from canine epiphyses contain approximately 1700 glucocorticoid receptor sites/cell.

Binding specificity analysis

The capacity of competitive ligand to displace [^{3}H]TA from the glucocorticoid receptor is illustrated in Fig. 3. Relative binding affinities were evaluated by assessing the molar concentration of competitive radioinert ligand required to displace 50% of the labeled ligand from the receptor. The relative binding affinities of steroid for

Table 1. DNA–cellulose binding of activated glucocorticoid receptor

	Nonactivated + MoO_4 (%)	Activated + MoO_4 (%)	Activated (%)
Mean	8.1 ± 3.6[a]	10.0 ± 4.1	46.2 ± 20.8
n	7	8	11

Analysis of [^{3}H]TA–receptor complex binding to DNA–cellulose. [^{3}H]TA (2.0×10^{-8} M) was incubated at 0–4°C for 18–24 h in the presence (nonactivated sample) or absence (activated sample) of 10 mM molybdate with the 85,000 *g* supernatant fraction of chondrocyte cytosol. Nonactivated cytosols and cytosols activated *in vitro* by warming to 25°C for 20 min were incubated with DNA–cellulose and allowed to equilibrate at 0–4°C for 1 h. After thorough washing, aliquots of the uniform suspension of DNA–cellulose with [^{3}H]TA–receptor complex bound were removed for scintillation counting. Total and nonspecific binding (in the presence of 100-fold molar excess of radioinert triamcinolone acetonide) were assayed in identical cytosolic aliquots utilizing HAP. Data are expressed as the percentage of specifically bound [^{3}H]TA–receptor complex bound to DNA–cellulose.
[a]Standard deviation.

receptor were found to be triamcinolone acetonide > corticosterone > hydrocortisone > progesterone > medroxyprogesterone acetate ≫ 17α-hydroxyprogesterone ≫ testosterone > 17β-estradiol.

Receptor binding to DNA–cellulose

Table 1 illustrates data reflecting the capacity of the glucocorticoid receptor complex activated *in vitro* to bind to DNA–cellulose. As is indicated, $46 \pm 21\%$ ($n = 11$) of the total ligand specifically bound to receptor (by hydroxyapatite) was capable of binding to DNA–cellulose. Conversely, steroid–receptor complex maintained in the presence of 10 mM molybdate at 0–4°C or activated *in vitro* in the presence of 10 mM molybdate failed to bind to DNA–cellulose to a significant extent, $8 \pm 4\%$ ($n = 7$) and $10 \pm 4\%$ ($n = 8$) respectively. These data confirm the capacity of receptor activated *in vitro* to bind effectively with DNA and also confirms the inhibitory effect of molybdate ion on the molecular mechanisms of activation.

Anion exchange chromatography

The chromatographic resolution of the activated and nonactivated complexes on DEAE–Sephadex anion exchange resin was utilized to further characterized the glucocorticoid binding protein. An elution profile of the nonactivated complex is presented in Fig. 4a. The cytosol was maintained throughout experimental manipulation under nonactivating conditions (0–4°C, 10 mM Na_2MoO_4). The receptor was eluted from anion exchange columns previously equilibrated with 10 mM molybdate by a 0–1.0 M KCl gradient also containing 10 mM molybdate. The nonactivated receptor form eluted from these columns at 0.4 M KCl. Void volume radioactivity was assayed for HAP binding, indicative of ligand–protein interactions and was found to be negative in this regard. The elution pattern of this nonactivated complex is similar to that described for the nonactivated hepatic glucocorticoid receptor Binder II [17].

The elution profile of the receptor activated *in vitro* by warming for 20 min at 25°C is illustrated in Fig. 4b. The high salt or nonactivated peak was significantly reduced following *in vitro* activation. The elution profile of the nonactivated form of the receptor was approximately 24% of the 0.4 M peak illustrated in Fig. 4a (2220 vs 529 mm^2). Activation apparently induced a shift in receptor form to the activated species as is evidenced by the appearance of the 0.2 M activated peak. The 0.2 M KCl peak was however approximately 18% of the nonactivated receptor form (2220vs 401 mm^2). Analysis of DNA–cellulose binding to the 0.2 M KCl peak fractions proved negative, possibly due to insufficient concentrations of radiolabeled receptor within these fractions. The void volume peak in Fig. 4b increased approximately 40% above that of the flow through volume in Fig. 4a (888 vs 2130 mm^2).

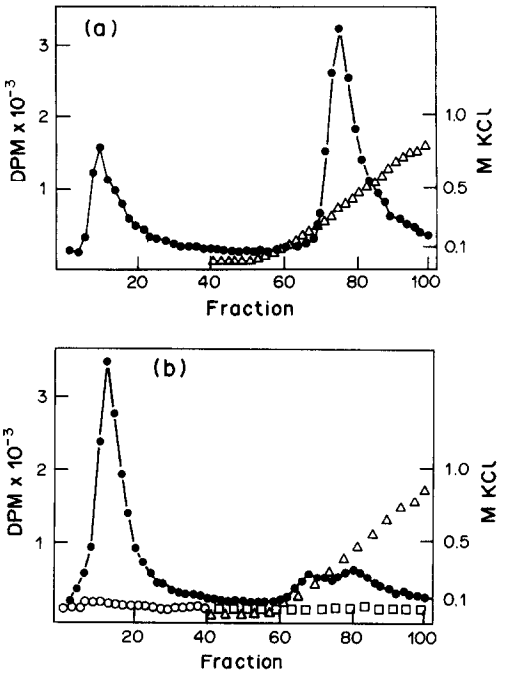

Fig. 4. DEAE–Sephadex elution profiles of nonactivated (a) and activated (b) [^{3}H]TA–receptor complexes from fetal rat epiphyseal chondrocyte cytosolic protein. Equal aliquots of the 85,000 *g* chondrocyte cytosolic fraction were labeled with 5.0×10^{-8} M [^{3}H]TA at 0–4°C for 18–24 h. Following incubation, the nonactivated cytosolic sample, containing 10 mM molybdate, was applied directly to DEAE–Sephadex columns with a layer of dextran-coated charcoal incorporated within. The activated sample, incubated without molybdate was heated to 25°C for 20 min and subsequently applied to similar columns. Eighty 0.8 ml fractions were eluted from the columns using a linear 0–1.0 M KCl gradient. Alternate samples were used to determine radioactivity (●) or for conductivity measurements (△) or to determine HAP (○) or DNA–cellulose (□) binding.

As such, the void volume peak fractions in the activated preparations were assessed for hydroxylapatite binding. Previous studies have equated increased radioactivity in void volume fractions following glucocorticoid receptor activation to formation of corticosteroid Binder 1B as described by Bastl *et al.*[34] and Carbone *et al.*[35]. The present results do not support the presence of Binder 1B in the void volume fractions in that increased binding of radiolabeled material to HAP was not evident.

Receptor activated in the presence of a protease inhibitor cocktail (designed by Loosfelt *et al.*[28] in order to maintain the monomeric form of the rabbit progesterone receptor) eluted from anion exchange columns in a manner identical to that illustrated in Fig. 4b. Thus, the cytosolic components inducing the alteration in receptor elution profiles and by inference receptor structure and/or ligand binding characteristics do not appear to be sensitive to a cocktail composed of pepstatin A, leupeptin, bacitricin and aprotonin.

Protease assay

Experiments were designed to demonstrate and evaluate the endogeneous proteolytic activity in chondrocyte cytosol. This degradative activity was assessed based on temperature and pH optimums of caseinase activity. In addition, since Ca^{2+}-dependent proteases have previously been shown to degrade the extrogen receptor of calf uterus [36], the progesterone receptor of chick oviduct [37] and the thymic glucocorticoid receptor [38] and since chondrocytes exist *in situ* in an environment replete with calcium, the inhibitory effect of EDTA on protease activity was also evaluated. A direct temperature dependent effect on caseinase activity was noted (data not presented). Proteolysis was minimal at 0°C, the percentage of TCA soluble [^{14}C]casein activity was 2.3% after 60 min of incubation. Caseinase activity increased in a temperature-dependent manner at 15 and 30°C where the percentage of TCA soluble material was 6.5 and 13% respectively following 60 min.

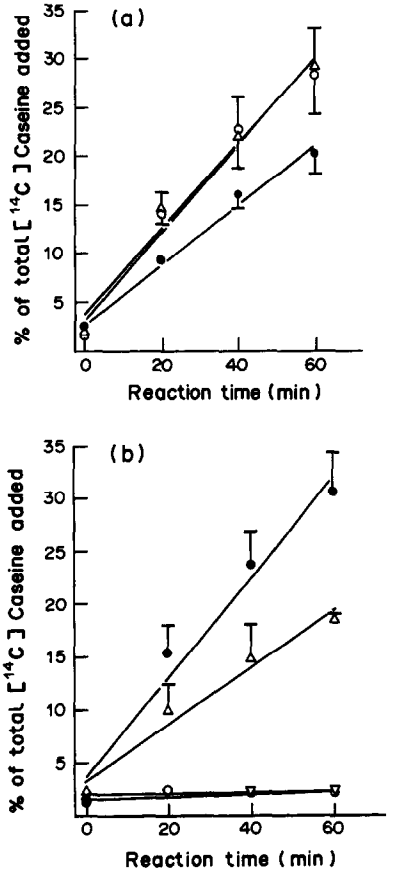

Fig. 5. Fetal rat chondrocyte cytosol [^{14}C]caseinase activity determination. The 85,000 *g* supernatant fraction of epiphyseal chondrocyte cytosol was incubated with a casein substrate mixture consisting of 0.2 μCi/ml [methyl-^{14}C]casein and 1.0 mg/ml unlabeled carrier casein. Following incubation at 30°C for 20, 40 or 60 min, the reaction was terminated with the addition of 10% trichloroacetic acid. Insoluble material was pelleted by rapid centrifugation and 50 μl aliquots of the supernatant were removed and the radioactive content in each sample was determined. Panel (a) illustrates pH-dependent reaction kinetics where reaction mixture pH was adjusted to 5.9 (△), 6.65 (○) and 7.4 (●). In panel (b) reaction conditions were optimized [30°C, pH 5.9 (●)] and the effects of 20 mM EDTA (△) and heat denaturation (▽) on reaction kinetics were evaluated. A control, monitoring [methyl-^{14}C]casein stability in the reaction mixture was run where buffer was substituted for cytosol (○). Each data point represents the mean ± standard deviation of 3 sample duplicates.

Concerning pH, optimum caseinase activity was noted at pH 5.9 and 6.65, where the percentage of TCA soluble [^{14}C]casein reached 28.4 and 28.3 of total [^{14}C]casein activity respectively following 60 m of incubation (Fig. 5a). Proteolytic activity was apparent at pH 7.4 where 20.1% of the total [^{14}C]casein activity was in the TCA soluble fraction at 60 min (Fig. 5a).

The analysis illustrated in Fig. 5b utilized optimal conditions for proteolysis as determined by previous experimental results (pH of 5.9 and an incubation temperature of 30°C). Optimized degradative capacity was illustrated as was the inhibitory effect on proteolytic activity of boiling the cytosolic preparation. These data thereby suggest that the degradative moiety in chondrocyte cytosol was proteinaceous in nature. In the samples containing heat inactivated cytosol, 2.2% of the radiolabeled casein activity was found in the TCA soluble fraction of the reaction mixture at 60 min. In a likewise fashion, casein degradation was minimal, equaling 2.3% of the total added label in samples in which reaction buffer was substituted for equal volumes of cytosol.

Also illustrated in Fig. 5b is the reduction in caseinase activity when the optimized reaction mixture (pH 5.9, 30°C) was made 20 mM with EDTA. At 60 min, 20 mM EDTA reduced the amount of TCA soluble [^{14}C]casein approximately 40% (18.6% of total [^{14}C]casein added) when compared to that reaction run without EDTA (30.5% of the total [^{14}C]casein added).

The preceding experiment demonstrated the inhibitory effect of 20 mM EDTA on proteolytic activity. In light of this inhibitory effect, experiments were conducted in which cytosol, pH 7.4 was activated *in vitro* in buffer containing 40 mM EDTA. The activated cytosol was subsequently applied to DEAE–Sephadex columns and subjected to gradient elution as described above. The elution profile in this instance was identical to that shown in Fig. 4b where the 0.4 M KCl peak was reduced, a small 0.2 M KCl peak was evident and an increase in radioactivity in the void volume was apparent. Thus, although EDTA was an effective inhibitor of caseinase activity and presumably receptor modification, it was apparently an ineffective inhibitor (at 40 mM) of the activation induced alterations in receptor structure/function.

Sucrose gradient ultracentrifugation

When chondrocyte cytosolic preparations were incubated with [^{3}H]TA in the presence of 10 mM

molybdate and subjected to sucrose gradient ultracentrifugation, a single binding moiety was noted. Using BSA and aldolase as standards, the sedimentation constant of the glucocorticoid binding component was $9.3 \pm 0.2\,s$ ($n = 6$) (Fig. 6a). When a 100-fold molar excess of radioinert competitor was added to the cytosolic mixture containing [^{3}H]TA and molybdate, the 9.3 s binding peak was significantly reduced in size (Fig. 6b). Cytosols were also subjected to *in vitro* activation both in the presence and absence of molybdate in order to evaluate the effects of this treatment on the sedimentation constant of the molecule. Figure 6c represents the sedimentation profile of cytosol activated *in vitro* in the presence of 10 mM molybdate. A single [^{3}H]TA binding component is evident with an s-value similar to that presented for the nonactivated receptor form maintained in the presence of molybdate. *In vitro* heat activation of the cytosol without molybdate in the buffer (Fig. 6d) resulted in the complete disappearance of the 9s glucocorticoid binding form.

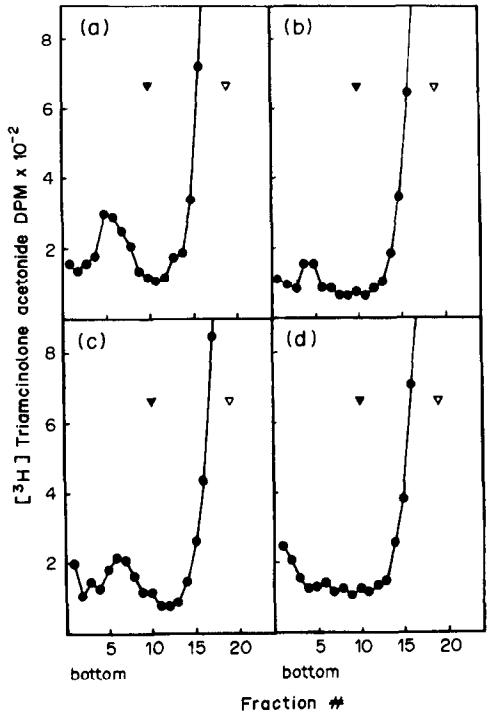

Fig. 6. Sucrose gradient sedimentation profiles of [^{3}H]TA bound to cytosolic protein from fetal rat epiphyseal chondrocytes. The 85,000 *g* supernatant fraction of chondrocyte cytosol was aliquoted into four equal fractions and incubated with [^{3}H]TA (5.0×10^{-8} M) at 0–4°C for 18-24 h. Panel (a), nonactivated cytosol incubated in the presence of 10 mM molybdate; panel (b), nonactivated cytosol incubated in the presence of 10 mM molybdate and a 100-fold molar excess of radioinert competitive ligand; panel (c), cytosol incubated in the presence of 10 mM molybdate and activated *in vitro* by warming to 25°C for 20 min; panel (d), cytosol incubated without molybdate and activated *in vitro* by warming. 0.1 ml aliquots of the cytosol were layered on linear 5–20% sucrose gradients and centrifugated at 149,000 *g* for 16 h. Following centrifugation the bottom of each gradient tube was pierced and 10 or 20 drop fraction were collected. Crystalline bovine serum albumin (bBSA, ▽) and aldolase (▼) were utilized as standards. Radioactivity or absorbence at 280 mμ was determined for each sample.

DISCUSSION

Data presented previously by Schmidt *et al.*[39] demonstrate a similarly "activation-labile" glucocorticoid receptor in a steroid resistant variant of CEM-C7 human lymphoid cells. Schmidt and colleagues noted that although the clone C7 and the subclone variant 4R4 exhibited similar K_ds for glucocorticoid binding to the receptor, the 4R4 subclone exhibited an increased *in vitro* activation induced lability of the glucocorticoid receptor. The increased lability of the receptor was evidenced by a time-dependent reduction in the high salt nonactivated peak eluting from DEAE–cellulose columns without a concomitant increase in the low salt or activated receptor form. These authors attribute the instability of the activated receptor form to denaturation or to dissociation of ligand from the receptor. We do not attribute the *in vitro* heat activation induced lability of the glucocorticoid receptor in chondrocyte cytosolic preparations to an aberrant receptor form as in the 4R4 subclone. The chondrocytes in the present studies were isolated from normal periparturitional fetuses. However, we do wish to emphasize the similarities in the data for the activation labile forms of the receptor on anion exchange columns.

The process of activation has been previously shown to allow sensitive regions of the glucocorticoid receptor to become more susceptible to proteolytic attack [40]. In light of the known susceptibility of steroid receptors to proteolysis, experiments were performed where a protease inhibitor cocktail consisting of pepstatin A, leupeptin, aprotonin and bactricin was added to the cytosol prior to *in vitro* activation. DEAE–Sephadex chromatographic profiles of this material were similar to those presented for chromatographic runs without inhibitor. These data indicate that although activated receptor lability may be a function of proteolytic attack, the enzymes responsible are not likely to include: pepsin, renin, cathepsin D, trypsin, chymotrypsin, papain, plasmin, thrombo-kinase, kallikrein, and cathepsin B, enzymes which have been shown to be inhibited by components of the protease inhibitor cocktail described above.

Vedeckis[40], hypothesizes that the structure of the activated glucocorticoid receptor is monomeric in nature but comprised of three distinct peptide moieties interspaced with protease susceptible regions. The intact 96 K receptor monomer is comprised of a 24 K meroreceptor/hormone binding component, a 29 K DNA binding region and a 43 K "modulating" domain which may serve to locate the receptor at the nuclear membrane or matrix, proximal to the location of specific DNA binding

sequences. Receptor subjected to limited proteolytic cleavage by chymotrypsin generates a 3.2 *s* 53 K receptor fragment. The 53 K receptor fragment is comprised of the 24 K meroreceptor and the 29 K DNA binding moiety. Importantly, the 53 K form retains the capacity to bind glucocorticoid and DNA–cellulose whereas the 24 K meroreceptor loses this DNA binding capacity. Trypsinization induces the formation of a 24 K hormone binding meroreceptor which does not bind DNA and a 72 K fragment comprised of the DNA binding and modulating domains. It should be noted in the present work, that although activation appeared to induce alterations in receptor structure/function as evidenced by altered DEAE–Sephadex chromatographic profiles, the receptor molecule retained its DNA–cellulose and ligand binding capacity when analyzed without prior chromatographic separation on DEAE–Sephadex. These data then suggest the formation of the 53 K hormone–DNA binding receptor fragment following activation. DEAE–Sephadex chromatography may be dependent on the cleaved 43 K modulating receptor fragment. Alternatively, the elution profiles of activated receptor illustrated in the present studies may be a resultant of further fragmentation of the 53 K form and/or enhance ligand dissociation.

Calcium activated proteases have been shown to cleave estrogen receptor from calf uterus [36], progesterone receptor from chick oviduct [37] and the chick thymic glucocorticoid receptor [41]. A ubiquitous neutral calcium dependent protease, calpain has been shown to induce conversion of the rat thymus glucocorticoid receptor to meroreceptor [38]. Additional evidence was presented by Bodwell and associates demonstrating the presence of a heat stable calpain inhibitory factor termed calpastatin. High calpastatin/calpain ratios were noted in tissue exhibiting glucocorticoid receptor stability, the converse relationship was evident in tissue cytosols where glucocorticoid receptor exhibited relative lability. Calpain activity has also been shown to be inhibited by 20 mM concentrations of EGTA (Bodwell, personal communication).

In light of the data concerning the proteolytic sensitivity of glucocorticoid receptor in certain tissue, the activity of endogenous proteases in chondrocyte cytosolic preparations was assessed using a [^{14}C]casein degradation assay. The inhibitory effect of 20 mM EDTA on caseinase activity was also evaluated. A temperature-dependent protease activity with a pH optimum of 5.9–6.65 has been demonstrated. This acidic protease activity was inhibited by boiling and by the addition of 20 mM EDTA.

Since EDTA has been shown to exert an inhibitory effect on calcium activated proteases, the chelating agent was evaluated for its capacity to inhibit receptor proteolysis based on DEAE–Sephadex profiles. 40 mM EDTA added to cytosolic preparations prior to *in vitro* activation, did not inhibit receptor modification as assessed by DEAE–Sephadex chromatography. The lack of an apparent effect of EDTA on preventing receptor modification may be a consequence of the high levels of calcium within chondroctye cytosol or may be a function of the relatively minute concentrations of receptor acting as enzymatic substrate. In this latter case, even minimal levels of active degradative enzymes may effectively proteolyze femtomolar concentrations of receptor.

Density gradient ultracentrifugation was utilized to further characterize the chondrocyte glucocorticoid receptor based on sedimentation coefficients and displacement of ^{3}H-ligand from peak fraction. Cytosolic preparations were also subjected to *in vitro* activation in the presence or absence of molybdate in order to assess the receptor stabilizing effect of this divalent anion. The gradient profiles were evaluated for susceptibility or resistance to activation induced receptor fragmentation. It was anticipated that through density gradient ultracentrifugation, receptor fragment size would be measurable and thus would provide some insight into the mechanism of receptor proteolysis.

The data indicate that the molybdate stabilized nonactivated receptor form sediments at approximately 9.3 *s*, results consistent for glucocorticoid receptor specifically [42, 43] and for steroid receptors in general [44]. The addition of radioinert competitor effectively displaced [^{3}H]TA from the 9.3 *s* binding component thus demonstrating receptor specificity. Molybdate was shown to inhibit the activation induced modification in glucocorticoid receptor sedimentation profile. These data are consistent with those presented previously demonstrating the inhibitory effect of molybdate on activation induced receptor transformation [17] and lability of glucocorticoid receptor [39]. Sedimentation analysis of the receptor, activated in the absence of molybdate, illustrate the labile nature of the chondrocyte cytosolic glucocorticoid receptor. No evidence of the 9.3 *s* peak was apparent in these elution profiles. In addition, no concomitant increase in 5 *s* receptor monomer or 3 *s* fragment were noted. The sedimentation profile of the activated receptor was once again indicative of a modification of receptor structure/function. This altered functional state was due perhaps to extensive proteolysis and/or ligand receptor dissociation. In regard to this latter supposition, the prolonged ultracentrifugation required in swinging bucket procedures may exacerbate activation induced ligand receptor dissociation and thus inhibit detection of receptor fragments.

In summary, we have characterized a cytosolic glucocorticoid receptor of fetal rat epiphyseal chondrocytes based on saturation, Scatchard and binding specificity analyses, DNA–cellulose binding capacity, anion exchange chromatographic profiles and sucrose density gradient analysis. Endogenous chondrocyte cytosolic proteolytic activity was also assessed in order to equate fragmentation of activated receptor with proteolytic capacity.

The results indicate that the glucocorticoid receptor in cytosolic preparations of chondrocytes isolated from fetal rat epiphyses is a single class of saturable, highly specific steroid binding component exhibiting a high-affinity, in the nanomolar range, for glucocorticoids. These data are in close agreement with those presented previously for a variety of cartilagenous tissue, species and methodologies [9, 13–16]. Data presented herein also demonstrates the functional capacity for receptor activation by demonstrating increased DNA–cellulose binding activity following *in vitro* activation. Elution profiles of the nonactivated receptor form from DEAE–Sephadex anion exchange columns were analogous to those presented previously for the nonactivated receptor form in a variety of tissue. Conversely, elution patterns of the activated receptor form were suggestive of receptor fragmentation and/or ligand–receptor dissociation. Sucrose gradient ultracentrifugation profiles corroborate the anion exchange data in that nonactivated receptor sedimented at 9.3 *s*, not unlike values presented for the classic glucocorticoid receptor Binder II and for steroid receptors in general. The activated receptor form exhibited gradient sedimentation profiles highly suggestive of receptor fragmentation and/or ligand receptor dissociation. Assessed cytosolic proteolytic potential based on caseinase activity was temperature-dependent and exhibited a moderately acidic pH optimum. The caseinase activity was proteinaceous in nature as evidenced by its susceptibility to heat denaturation. The caseinase activity was also shown to be significantly inhibited by 20 mM EDTA. Although the caseinase activity was inhibitable by 20 mM EDTA, comparable levels of EDTA failed to prevent receptor modification as assessed by anion exchange chromatography. The data indicate that the glucocorticoid receptor in chondrocyte cytosol is functional in its capacity to bind to DNA following specific ligand binding and subsequent activation. The receptor is however highly susceptible, following *in vitro* activation to proteolytic activity derived from endogenous sources.

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