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AUTORADIOGRAPHIC STUDY OF THE NUCLEAR TRANSFER OF THE ANTIGLUCOCORTICOID COMPOUND RU38486 IN MOUSE TISSUES

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Summary—In order to investigate the mode of action of RU38486, a recently described antiglucocorticoid molecule, we have studied by autoradiography the localization of [^{3}H]RU38486 in various glucocorticoid target tissues 30 and 60 min following *in vivo* injection. The results obtained in brain, liver and kidney sections clearly demonstrate that [^{3}H]RU38486 is specifically translocated in target cell nuclei. Indeed, the nuclear labelling observed after injection of the tracer was significantly decreased after injection of [^{3}H]RU38486 in the presence of a 100-fold excess of unlabelled RU38486. The distribution of the specific labelling of RU38486 within the central nervous system was very similar to that previously described for dexamethasone with an accumulation of radioactivity in the cell nuclei of the hypothalamic area of the nucleus arcuatus. Our results thus provide additional evidence for an action of this antiglucocorticoid molecule at the nuclear level.

INTRODUCTION

Despite extensive investigation, the mode of action of antiglucocorticoid compounds is still poorly defined. It is now generally accepted that these molecules exert their action by competing with the hormonal steroids for cytosolic receptors, but it remains to be determined whether or not the antagonist–receptor complexes transfer to the nucleus and bind to nuclear acceptor sites. Experiments performed using weak antiglucocorticoid molecules such as cortexolone, medroxyprogesterone acetate, promegestone as well as several derivatives of progesterone, have provided contradictory results [1–6]. More recently, several groups have described the properties of a new synthetic molecule (RU38486), which binds with a high affinity to glucocorticoid receptors and exhibits potent antagonistic effects with almost no agonistic activity at high concentrations [7–11]. Studies of the nuclear transfer of this compound led however to contradictory results. Jung-Testas and Baulieu[7] provided evidence in cultures of L_{929} fibroblasts that [^{3}H]RU38486 was translocated into the cell nucleus, although less efficiently than dexamethasone. A similar observation was also made by Chasserot-Golaz and Beck in hepatoma cells [11]. In contrast, Moguilewsky and Philibert[9] failed to show any significant nuclear transfer of the antagonist molecule into rat thymus lymphocytes. One possible explanation for these discrepancies may lie in the different methods used to determine nuclear binding. As recently stated by Kaufmann and coworkers [12], the conventional techniques which imply cell disruption, centrifugation and triton treatment may lead to erroneous determinations in some types of cell. In order to circumvent these difficulties we have studied in the present work the autoradiographic localization of [^{3}H]RU38486 in several glucocorticoid target tissues following *in vivo* injection. This method has been extensively used in the past to determine the localization of glucocorticoid receptors in brain cell nuclei as well as in other tissues [13–18].

EXPERIMENTAL

Animals and reagents

Female C57 BL/6 mice, 6–8-weeks old, were adrenalectomized under pentobarbitone anesthesia 1 week before each experiment and were given free access to standard diet and 0.9% saline. [6,7 ^{3}H]RU38486 (17-βhydroxy-11,4-dimethyl aminophenyl-17 β-propynyl estra 4,9 diene-3-one) [37 Ci/mmol] and unlabelled RU38486 were kindly provided by Roussel-Uclaf (Romainville, France). [1,2,6,7 ^{3}H]corticosterone (103 Ci/mmol) was purchased from Amersham Centre (U.K.). Unlabelled corticosterone was from Sigma.

Autoradiographic procedures

Anesthetized mice were injected with 100 μl of radioactive solutions via the jugular vein. Seven mice received 20 μCi ($\sim$1.3 μg/100 g bwt) of [^{3}H]RU38486 alone (group I) and 5 mice were injected with the same quantity of [^{3}H]RU38486 in presence of a 100-fold excess of unlabelled RU38486 (group II). Animals were killed by decapitation either 30 min after the injection (4 mice of each group) or 1 h after the injection (3 mice of group I and 1 mouse of group II). The brain, kidneys and liver were quickly removed and frozen in liquid nitrogen–cooled isopentane.

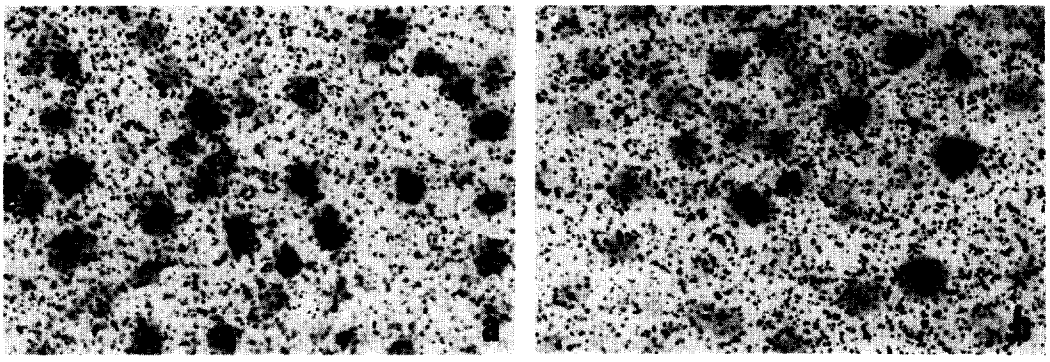

Fig. 1. Autoradiograms from mouse liver of (a) [^{3}H]RU38486 alone and (b) [^{3}H]RU38486 with a 100-fold excess of unlabelled RU38486 (× 800). The cell nuclei appear dark as photomicrographs were taken using both orange and pink filters.

The thaw-mounted autoradiographic method used has been previously described [17]. Briefly, sections of about 8 μm thickness were made by means of a cryostat (American optical, type Cryocut) maintained at a temperature of −18°C. For the brain, only sections containing target brain regions for [^{3}H]dexamethasone and [^{3}H]corticosterone (respectively, the medial hypothalamus and various regions of the limbic system) were studied.

The sections were transferred in the dark onto slides precoated with Kodak AR 10 stripping film. After about 4 months of exposure at a temperature of −20°C, autoradiograms were developed and stained with methyl-green pyronin.

Evaluation of the cell concentration of radioactivity

The cell nuclear and cytoplasmic densities of silver grains were determined by counting grains present in a surface area of 25 μm^2 corresponding to a square of a micrometer placed in a microscope eyepiece at a magnification of × 1000. For the cell nuclear density a square of a micrometer was placed over the center of the cell nucleus, whereas for the cytoplasmic density, when possible, several areas of 25 μm^2 were considered and the mean value determined. The density of silver grains outside the sections was negligible.

Liver and brain

For each chosen section, the nuclear and cytoplasmic densities of silver grains of all the cells present within the total surface of the micrometer (2500 μm_2) were determined. The quantitative evaluation of the cell grain densities in the brain were made only in two hypothalamic areas of the nucleus arcuatus.

Kidney

Within the renal cortex, 3 different structures were considered: glomeruli, tubules with cell cytoplasm highly stained with pyronin (presumably proximal tubules) and tubules lightly stained with pyronin (presumably distal tubules). As for the renal medulla, sections of nephron segments were randomly taken for quantitative evaluation. In each chosen section, all the cells of 4 of each of these various structures were considered. We quantified silver grains in 2 sections of liver, brain and kidney from 3 animals, which received [^{3}H]RU38486 alone and 3 injected with [^{3}H]RU and an excess of unlabelled RU38486.

Statistical analysis

Student t-test was used to establish the significance of the difference between mean values.

Biochemical analysis

Anesthetized mice were injected with 100 μl of radioactive solution via the jugular vein. Three mice received 20 μCi of [^{3}H]corticosterone alone (group I), 6 mice received 20 μCi of [^{3}H]corticosterone with a 50-fold excess of either unlabelled RU38486 (group II, 3 mice) or unlabelled corticosterone (Group III, 3 mice).

Animals were killed by decapitation 30 min after the injection. The brain was removed quickly, the hippocampal region dissected and homogenized using a polytron in ice cold incubation medium (Na^+ 133 mM, K^+ 6 mM, Ca^{2+} 1 mM, Mg^{2+} 0.5 mM, Cl^- 136 mM, $H_2PO_4^-$ 6 mM, Tris–HCl 5 mM, glucose 5 mM, pH 7.4). The homogenates were then centrifuged for 30 min at 105,000 ***g***. Aliquots of the cytosol (100 μl) were treated by 200 μl of dextran coated charcoal for 15 min at 4°C. After 2 min centrifugation at 10,000 ***g***, the protein bound radioactivity was determined by scintillation spectrometry and expressed as fmol/mg protein. Protein determination was made according to Lowry using bovine serum albumin as standard.

RESULTS

Liver

After injection of [^{3}H]RU38486 alone, hepatic tissue was highly labelled although the radioactivity was not uniformly distributed (Fig. 1a). In animals injected with the tracer and a 100-fold excess of unlabelled

RU38486, the cell nuclear density of silver grains was significantly diminished, whereas the cytoplasmic radioactivity remained unaltered (Figs 1b and 2).

Brain

Autoradiograms from mice injected with [^{3}H]RU38486 alone showed almost no radioactivity within the brain parenchyma. However a high nuclear labelling was observed in about 25% of the cells of the nucleus arcuatus hypothalami (Figs 3a, b and 4).

No other brain areas contained significant accumulation of nuclear radioactivity as illustrated by autoradiograms taken from a well known target area for [^{3}H]corticosterone, the gyrus dentatus (Fig. 3c). In the presence of an excess of unlabelled RU38486, the preferential accumulation of radioactivity within cell nuclei of the nucleus arcuatus hypothalami was abolished (Figs 3d and 4).

Kidney

The intensity of the radioactivity within the renal cortex was higher than that of the medulla (Figs 5a and 5b). Although the nuclear silver grain density greatly varies from one cell to another, the majority of the tubules highly stained with pyronin (proximal tubules) were more intensely labelled than glomeruli or than the distal tubules (lightly stained with pyronin, Figs 5a and 5c).

After injection of [^{3}H]RU38486 with an excess of unlabelled RU38486, the nuclear density of radioactivity was diminished in all the renal structures whereas the cytoplasmic radioactivity remained unchanged (Figs 5e, f and 6). In addition, no significant difference was observed between autoradiograms obtained from mice killed 30 or 60 min after the injection of the radioactive solution (results not shown).

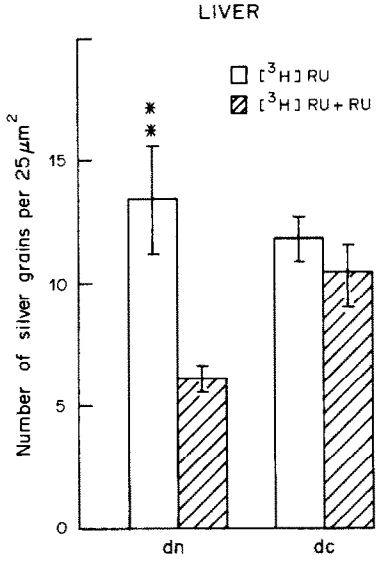

Fig. 2. Quantitative evaluation of radioactivity within hepatocytes after injection of [^{3}H]RU38486 alone and in presence of a 100-fold excess of unlabelled RU38486. Each column represents the mean value ± SEM of the nuclear (dn) and the cytoplasmic (dc) grain densities obtained from 4 different animal of each experimental group. $^{**}P < 0.01$.

Biochemical analysis

From the results presented in Fig. 7 it appears that unlabelled RU38486, when added in a 50-fold excess is able to significantly displace [^{3}H]corticosterone from its binding sites in hippocampal region of mouse brain. Although the efficiency of RU38486 is inferior to that of unlabelled corticosterone itself, these results suggest that this compound is readily transported across the brain–blood barrier as also suggested by the results of Gaillard and coworkers [20].

DISCUSSION

Our results show that the antiglucocorticoid compound RU38486 accumulates in the cell nuclei of several well characterized glucocorticoid target tissues. This nuclear retention may be considered as specific since it is markedly diminished in animals receiving an excess of unlabelled steroid together with the tracer. We failed to show any displacement of cytosolic radioactivity in the presence of unlabelled competitors in accord with previous studies [16–19].

One can argue that RU38486 also binds with a high affinity to progesterone receptors [21, 22] and that the binding we observed could be to those receptors rather than to glucocorticoid receptors. When considering however the brain localization of radioactivity it appears that the distribution of the nuclear retention of [^{3}H]RU38486 closely resembled that described previously for [^{3}H]Dexamethasone. In addition, several authors have studied the localization of progesterone receptors in the brain using the synthetic tracer R5020, and showed that they are almost 10-fold more abundant in the pituitary than in the hypothalamus [23, 14]. The fact that [^{3}H]RU38486 did not accumulate within the hippocampal cell nuclei as did corticosterone, was not due to its inability to reach these cells since we showed that this compound was able to compete for [^{3}H]corticosterone binding in hippocampal cytosol. This suggests that the hippocampal concentration of [^{3}H]RU38486 is inferior to that of nucleus arcuatus hypothalami and is below the threshold required for autoradiographic demonstration.

Similarly, it is known that glucocorticoids may bind to mineralocorticoid receptors in the kidney but RU38486 has been demonstrated to have almost no affinity for mineralocorticoid receptors [22], thus suggesting that the tracer used in this study is mainly bound to glucocorticoid receptors. We also present evidence suggesting that radioactivity accumulates in cell nuclei of the proximal tubules as compared to distal tubules, glomeruli and the medullary cells. Given the techniques used in this study it is however not possible to give a more detailed description of the

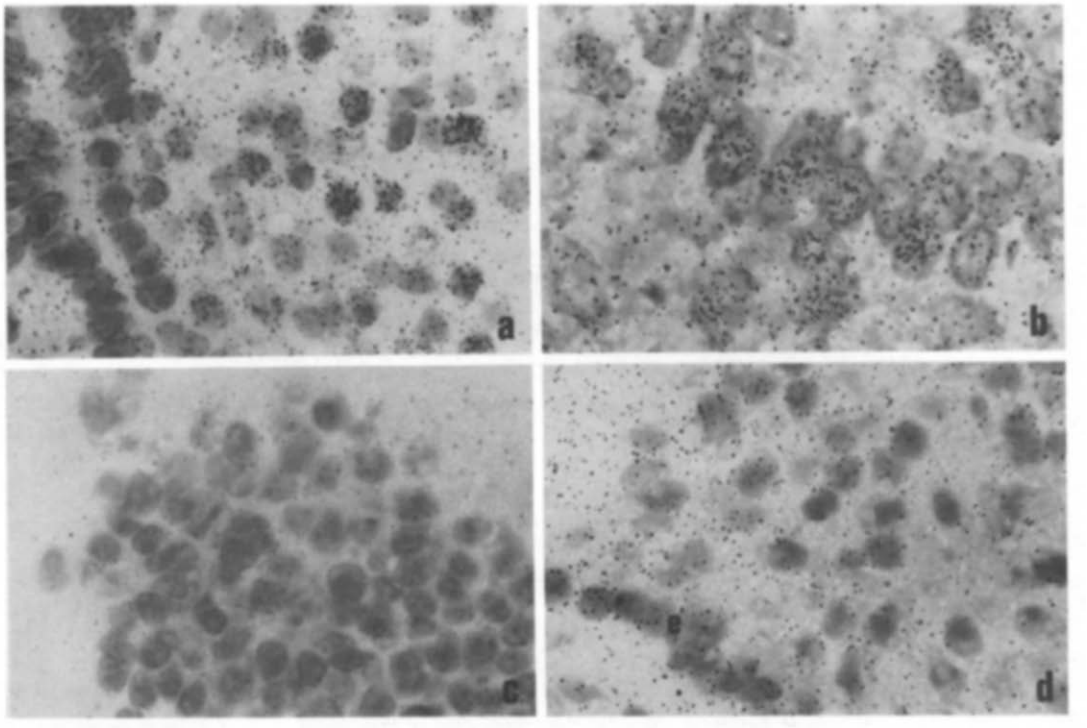

Fig. 3. a–c. Autoradiograms from mouse brain after injection of [^{3}H]RU38486 alone. a. Nucleus arcuatus hypothalami (× 540). (The photomicrographs were taken using orange and pink filters, the cell nuclei appear dark). b. Nucleus arcuatus hypothalami (× 990). (No filter was used, the cell cytoplasm appears dark). c. Gyrus dentatus (× 540). d. Autoradiograms from nucleus arcuatus hypothalami after injection of [^{3}H]RU38486 with an excess of unlabelled RU38486 (× 540). c–d. Orange and pink filters were used.

distribution of the receptors along the nephron. Such a study would probably require another approach namely that used by Farman *et al.* to investigate aldosterone binding sites in isolated tubules [24,25].

Our study indicates that the antiglucocorticoid molecule RU38486 is translocated into the cell nucleus but does not shed light on the mechanism of its action. In their experiments performed on rat thymic lymphocytes, Moguilewsky and Philibert have shown that the interactions of RU38486 with cytosolic receptors followed by heat activation led to the formation of complexes with an increased rate of dissociation [9]. They suggested therefore that this impaired activation process might explain their inability to demonstrate nuclear uptake. Although we have not in the present paper made time course experiments, we have no indication in favour of a rapid dissociation of the RU38486–receptor complexes. Indeed, the autoradiograms from animals killed 60 min after tracer injection were not different from those obtained after 30 min. Moreover, we have previously shown [10] that the affinity of RU38486 for dexamethasone binding sites is almost identical when measured at 4 and 37°C, whereas that of compound such as progesterone which dissociates rapidly from glucocorticoid binding sites is significantly higher at 4 than at 37°C.

Finally it should be considered that the results demonstrated here for RU38486 do not necessarily apply for all the antiglucocorticoid steroids. Indeed Coutard and coworkers have previously shown, using the same autoradiographic method, that cortexolone which exhibits *in vitro* anti-glucocorticoid

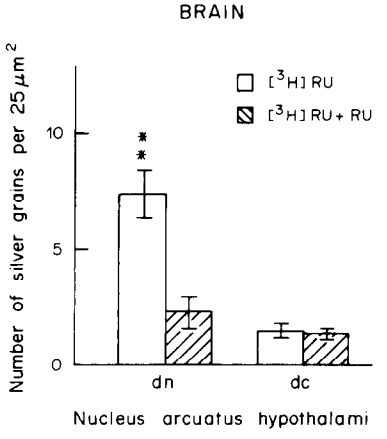

Fig. 4. Quantitative evaluation of radioactivity within cells from the hypothalamic area of the nucleus arcuatus after injection of [^{3}H]RU38486 alone and in presence of a 100-fold excess of unlabelled RU38486. Each column represents the mean value ± SEM of the nuclear (dn) and the cytoplasmic (dc) grain densities obtained from 3 different animals of each experimental group. $^{**}P < 0.01$.

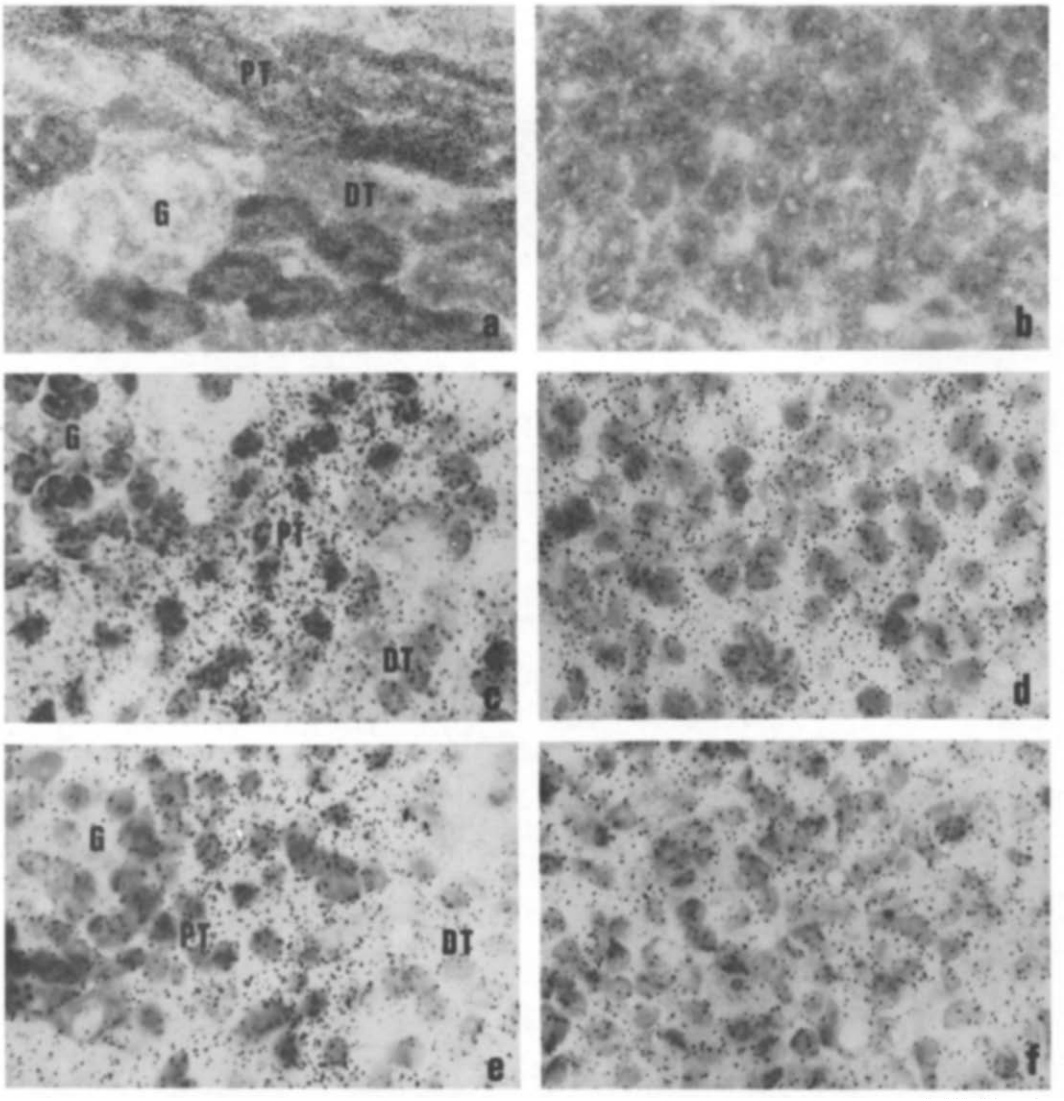

Fig. 5. Autoradiograms from mouse kidney. *a–d after injection of* [^3H]*RU*38486 *alone.* (a) Cortex: note that proximal tubules (PT) accumulate more radioactivity than distal tubules (DT) and glomeruli (G) (×340). (b) Medulla (×340). (c) Cortex: note large numbers of silver grains within the nucleus and the cytoplasm of the majority of the cells from proximal tubules compared to distal tubules and glomeruli (×680). (d) Medulla (×680). *e–f after injection of* [^3H]*RU*38486 *with a* 100*-fold excess of unlabelled RU*38486. (e) Cortex: note the marked decrease in nuclear labelling of the cells from the proximal tubules (×680). (f) Medulla (×680). c–f. The cell nuclei appear dark as photomicrographs were taken using both orange and pink filters.

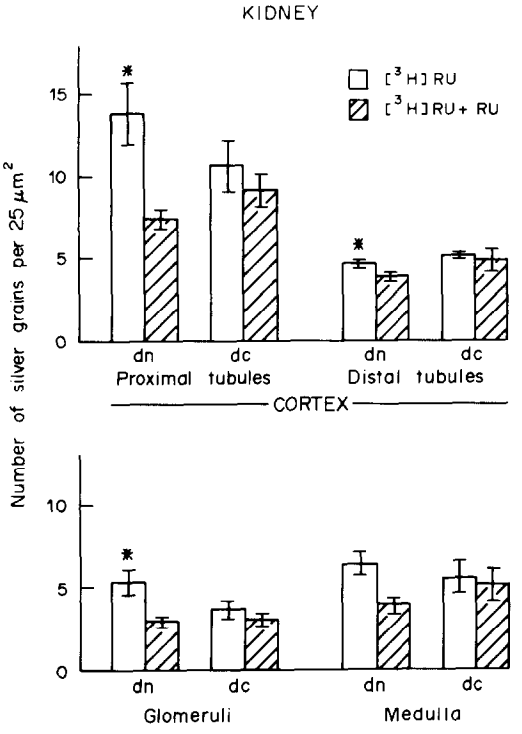

Fig. 6. Quantitative evaluation of the radioactivity within cells from several cortical structures: proximal tubules, distal tubules, glomeruli and sections of nephron, segments within the medulla, after injection of [^{3}H]RU38486 alone and in presence of a 100-fold excess of unlabelled RU38486. Each column represents the mean value of ±SEM of the nuclear (dn) and cytoplasmic (dc) grain densities from 3 different animals of each experimental group. $*P < 0.05$.

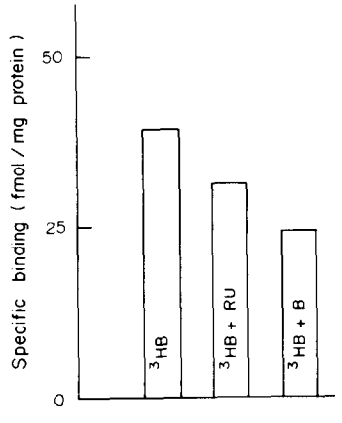

Fig. 7. Binding of [^{3}H]corticosterone in the cytoplasm of cells of the hippocampus: competition by unlabelled corticosterone and RU38486. 30 min following i.v. injection of 20 μCi of [^{3}H]corticosterone with or without a 50-fold excess of unlabelled competitor, the hippocampus was removed from 3 animals in each group, homogenized and centrifuged. Aliquots of the supernatants were then analyzed for protein-bound radioactivity. Each value expressed as fmol/mg protein represents the mean of triplicate determination in a given experiment. The total cytosolic radioactivity was almost identical in the 3 groups: 11,278 dpm/mg protein, 9,300 dpm/mg protein and 11,250 dpm/mg protein for groups I, II and III respectively.

activity [5, 6] does not accumulate in nuclei of brain glucocorticoid target cells [18]. This suggests that antagonist compounds may well exert their effects at different steps of the mechanism of steroid hormone action.

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