

Acute Glucocorticoid Pretreatment Suppresses Stress-Induced Hypothalamic-Pituitary-Adrenal Axis Hormone Secretion and Expression of Corticotropin-Releasing Hormone hnRNA but Does Not Affect *c-fos* mRNA or Fos Protein Expression in the Paraventricular Nucleus of the Hypothalamus

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Abstract

Corticosterone regulates both basal and stress-induced hypothalamic-pituitary-adrenal (HPA) axis activity in a negative-feedback fashion. However, the cellular and molecular mechanisms of this negative feedback have yet to be explicitly characterized. By comparing stress-induced *c-fos* and corticotropin-releasing hormone (CRH) expression in the paraventricular nucleus (PVN), we may be able to determine whether acute glucocorticoid treatment affects the net neural excitatory input to the PVN (represented primarily by *c-fos* mRNA expression) or directly affects the ability of cells in the PVN to respond to that input (represented primarily by CRH hnRNA expression). In the following studies, we observed the effect of acute glucocorticoid (RU28362) treatment on subsequent HPA axis reactivity by measuring stress-induced plasma hormone concentration [corticosterone and adrenocorticotropic hormone (ACTH)] and gene expression (*c-fos* and CRH) in the PVN. First, we examined the dose–response relationship between systemically administered RU28362 (1–150 µg/kg, i.p.) and suppression of the stress-induced corticosterone response. We then confirmed central nervous system access of the maximally suppressive dose of RU28362 (150 µg/kg) by an *ex vivo* radioligand binding assay. RU28362 selectively occupied the majority of glucocorticoid receptors in the hippocampus and hypothalamus while having no effect on mineralocorticoid receptors. In separate studies, RU28362 (150 µg/kg) and corticosterone (5 mg/kg) were injected i.p. 1 h before restraint stress. Compared to vehicle-treated controls, rats treated with RU28362 and corticosterone had substantially blunted stress-induced corticosterone and ACTH production, respectively. Furthermore, treatment with RU28362 significantly blunted stress-induced CRH hnRNA expression in the PVN. By contrast, neither RU28362 nor corticosterone treatment had an effect on stress-induced neuronal activation as measured by *c-fos* mRNA and its protein product in the PVN. This dissociation between *c-fos* and CRH gene expression suggests that glucocorticoid suppression of HPA activity within this time-frame is not a result of decreased excitatory neural input to the PVN, but instead depends on some direct effect of RU28362 on cells intrinsic to the HPA axis.

Activity of the hypothalamic-pituitary-adrenal (HPA) axis is tightly regulated by the levels of glucocorticoid hormone secretion that vary with time of day and absence or presence of stressors (1). Chronic glucocorticoid hypersecretion, that may result from impaired glucocorticoid negative feedback, can have a wide range of adverse consequences, including dysregulation of other endocrine systems, immunodeficiency, cardiovascular disorders, energy metabolism disorders, neurotoxicity, cognitive dysfunction and mood alterations (1–3).

The mechanisms of glucocorticoid negative feedback have yet to be comprehensively determined. The ability of glucocorticoids to tonically suppress basal HPA axis activity, as well as to constrain stress-induced HPA axis activity, has been well documented (4). Glucocorticoid regulation of basal and stress-induced HPA activity may depend on both the integrated levels of glucocorticoids that the organisms are exposed to over a 24-h period (5), as well as the recent circadian pattern of glucocorticoid secretion (6). In addition, the stress-induced phasic increase in

glucocorticoid secretion may contribute to stress reactivity of the HPA axis (7, 8). In each of these cases, the cellular and molecular mechanisms underlying glucocorticoid regulation of the HPA axis remain poorly understood. Some of these regulatory actions are undoubtedly a result of direct effects of glucocorticoids on cells intrinsic to the HPA axis. However, glucocorticoids can also indirectly affect the HPA axis by modifying the activity of cells that provide neural (9, 10) and, perhaps, hormonal (11) input to the cellular components of the HPA axis. At a molecular level, these effects may be mediated by mineralocorticoid receptors (MR) or glucocorticoid receptors (GR), two members of the nuclear hormone receptor family, or possibly by a putative membrane bound steroid receptor(s) (12, 13). Moreover, in the case of MR and GR, the glucocorticoid effects may depend on direct modulation of gene expression via receptor interaction with a glucocorticoid response element (GRE), indirect modulation of gene expression by receptor interaction with other transcription factors, or modulation of cellular function independent of gene expression (e.g. altered ion channel activity, kinase activity) via other receptor protein-protein interactions (14–16).

We have found that a systemic injection of rats with the selective GR agonist RU28362 substantially blunts an HPA axis response to restraint administered 1 h later (17). GR is expressed abundantly throughout the brain but is found in especially high concentrations in the corticotropin-releasing hormone (CRH) neurones of the medial parvocellular portion of the paraventricular nucleus (PVN) and in corticotrophs of the anterior pituitary (2). Of the two types of glucocorticoid receptors, the lower affinity receptor, GR, is thought to mediate attenuation of HPA axis activity during stress, and also appears to be the only glucocorticoid receptor type expressed in the PVN under normal conditions (2, 18).

In the studies presented here, we compare the stress-induced expression patterns of the *c-fos* and CRH gene in the PVN to explore whether the inhibitory effect of RU28362 on HPA axis activity is due to a direct or indirect effect on cellular components of the HPA axis. In neurones, transynaptic events that result in depolarization, increased intracellular Ca^{2+} , or mitogen-activated protein kinase activation also rapidly induce *c-fos* expression within 15 min (19). A wide range of stressors, including restraint, immobilization, swimming, audiogenic noise and immune challenge, have been found to reliably induce *c-fos* expression in a variety of brain areas, and a basic neural map of the mammalian stress response is emerging from these studies (20–25). Not surprisingly, one brain region that consistently shows a stress-induced increase in *c-fos* expression is the PVN. Double-labelling studies have shown that the majority of cells in the PVN that express *c-fos* in response to stressful stimuli also express CRH (26, 27).

The magnitude of the *c-fos* response in the PVN generally correlates well with the magnitude of the HPA axis response, as measured by adrenocorticotrophic hormone (ACTH) and corticosterone secretion (23). Thus, the magnitude of *c-fos* expression in the PVN may reflect the recent relative net excitatory input to this brain region. If RU28362 pretreatment has an inhibitory effect on this input, then one would expect a corresponding decrease in the level of *c-fos* mRNA expression in the PVN. There is also the possibility that RU28362 may have a direct effect on *c-fos* expression within the PVN. However, *in vitro* studies suggest that if glucocorticoids have a direct effect on *c-fos* expression, it

may be stimulatory rather than inhibitory effect (28). However, the danger of extrapolating results from one cell type to another should also be considered.

Another gene that is rapidly induced by stress within the PVN is the CRH gene. The rapid induction of this gene is not apparent when measuring the relatively high constitutive levels of CRH mRNA expression (29). However, this rapid gene induction is evident when measuring the very short-lived intron containing CRH primary transcript (CRH hnRNA) (30). Expression of the CRH gene is not only induced by acute stress, but also is suppressed by glucocorticoids, presumably by a GRE-independent mechanisms involving GR interactions with other proteins that are critical for CRH gene expression (31).

A direct comparison of *c-fos* and CRH gene expression within the PVN may provide insight into common or differing signal transduction pathways that are modulated by stress or glucocorticoids. The majority of studies examining glucocorticoid regulation of *c-fos* and CRH gene expression have focused on the tonic regulation of this gene in the rat as a result of long-term removal or elevation of glucocorticoid levels. Here, we present studies examining the effect of acute RU28362 treatment on stress-induced *c-fos* and CRH gene expression in the PVN. As part of this examination, we characterized the dose–response relationship between RU28362 pretreatment and the corticosterone response to restraint. We also used an *ex vivo* glucocorticoid-receptor binding assay (32) to demonstrate that the dose of RU28362 used for our gene expression studies gains access to the brain and selectively occupies GR. We also compared the effect of RU28362 and corticosterone (which activates both MR and GR) on stress-induced Fos protein expression. Finally, we used *in situ* hybridization to examine both *c-fos* mRNA and CRH hnRNA expression in the PVN under our experimental conditions.

Materials and methods

Subjects

Young adult male Sprague-Dawley rats (Harlan, San Diego, CA, USA) weighing 250–350 g were housed in pairs in polycarbonate tubs (47 cm × 23 cm × 20 cm) in a sound-proof colony room maintained on a 12:12 h light/dark cycle (lights on at 07.00 h). Standard rat chow and water were provided *ad libitum*. Rats were given at least 2 weeks to acclimate to the colony before experimentation. All rats were weighed and marked on the day before experimentation.

Drug pretreatment

The dose of RU28362 chosen for these experiments was based on a preliminary dose–response experiment (Fig. 1). Rats were injected i.p. with varying doses (0, 1, 10, 25, 75, 150 µg/kg) of RU28362 1 h before 1 h of restraint ($n = 4–8$). Blood samples were taken via the tail vein at 0, 30, 60 and 120 min following the onset of restraint. Rats were briefly placed back in the restrainer to obtain post-stress blood samples. The lowest dose which effectively blunted the HPA axis response to restraint stress was 25 µg/kg. The dose of 150 µg/kg was chosen for subsequent studies to ensure that the pretreatment would have a maximal inhibitory effect on corticosterone secretion. RU28362 (150 µg/kg, a gift of the former pharmaceutical company Roussel Uclaf, France) and corticosterone (5 mg/kg, Steraloids Inc. Newport, RI, USA) were dissolved in 0.9% saline + 40% 2-hydroxypropyl-β-cyclodextrin (HBC, H107, Research Biochemicals, Natick, MA, USA).

Restraint stress

Rats were placed in a clear Plexiglas tube (23.5 cm in length and 7 cm in diameter) with tails protruding. The size of the tube restricted both lateral and forward/backward movement but did not interfere with breathing.

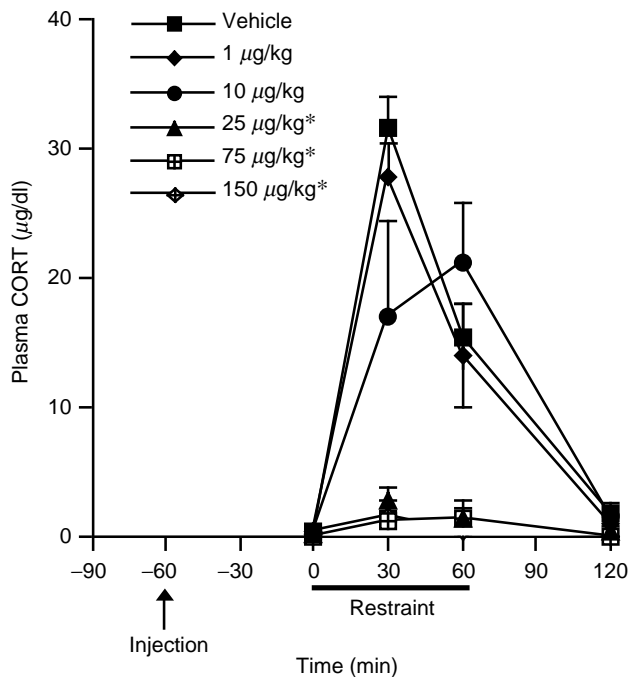


Fig. 1. RU28362 produced a dose-related decrease in stress-induced plasma corticosterone (CORT) ($\mu\text{g/dl}$) levels. Key includes concentrations of RU28362 injected i.p. 1 h before restraint onset. *Denotes doses that caused a significant ($P < 0.05$) reduction in stress-induced plasma corticosterone concentrations at 30 and 60 min after the onset of restraint ($n = 4-8$).

Experimental procedures

Ability of peripherally administered RU28362 to occupy corticosteroid receptors in brain

The first experiment was conducted to assess the ability of peripherally administered RU28362 (150 $\mu\text{g/ml}$) to selectively occupy GR in the hypothalamic region and hippocampus. All rats were bilaterally adrenalectomized under general anaesthesia, xylazine (10 mg/kg) and ketamine (50 mg/kg) mixture, to remove interference from endogenous corticosterone. Rats were injected 24 h later with either RU28362 (150 $\mu\text{g/kg}$) or vehicle. One hour after injection, rats were decapitated. The hippocampal formation and diencephalic block containing predominantly hypothalamus were rapidly removed, frozen over dry ice and stored at -80°C until assayed for corticosteroid-receptor binding levels (described below).

Effect of RU28362 and corticosterone pretreatment on Fos expression following restraint stress

The effect of RU28362 or corticosterone on Fos expression following restraint stress was determined in separate experiments by quantifying Fos protein immunoreactivity following immunohistochemical procedures described below. Rats were injected i.p. with RU28362 (150 $\mu\text{g/kg}$) or vehicle 1 h before either being exposed to 1 h restraint stress or left undisturbed in their homecage environment. Blood samples were collected after 30-min restraint via tail vein from subjects in restrainers by removing approximately 1–2 mm of the tip of the tail and stroking caudally into a microfuge tube pretreated with heparin. Plasma was stored at -20°C until time of corticosterone assay (described below). One hour after the termination of restraint stress, rats were deeply anaesthetized with a xylazine (20 mg/kg) and ketamine (100 mg/kg) mixture and perfused transcardially with 400–500 ml 0.01 M phosphate-buffered saline (PBS) followed by 400–500 ml of ice-cold 4% paraformaldehyde in 0.1 M PBS. Timepoint of perfusion was determined to be around the time of peak Fos expression in the PVN after restraint (20). Brains were removed and continued fixing in 4% paraformaldehyde for 24 h. Successive coronal sections (40 μm) at brain areas of interest were cut on a vibratome and collected in PBS. Examination of the effects of RU28362 on Fos expression was divided into four separate cohorts of rats with a final sample size for each treatment group of $n = 13-15$. A separate experiment following the same procedure was conducted examining the effect of corticosterone (5 mg/kg) or vehicle on Fos expression ($n = 4$). For this experiment plasma was stored at -80°C until time of ACTH assay (see below).

Effect of RU28362 pretreatment on *c-fos* mRNA and CRH hnRNA expression following restraint stress

Rats were injected with RU28362 (150 $\mu\text{g/kg}$, i.p.) or vehicle 1 h before the onset of restraint stress. Rats were exposed to either 15 min ($n = 6$) or 30 min ($n = 4$) of restraint stress. Trunk blood was collected into ethylenediaminetetraacetic acid (EDTA) coated tubes immediately following rapid decapitation. Time of sacrifice was selected based on peak expression levels in the PVN after restraint determined by timecourse studies conducted in our laboratory (unpublished data) and are consistent with published reports (29). Following rapid decapitation of rats, brains were quickly removed, frozen in isopentane chilled to -35°C and stored at -80°C . Sections, 10 μm thick, were cut on a cryostat (1850, Leica, Rijkswijk, the Netherlands), thaw mounted onto polylysine coated slides, and stored at -80°C until *in situ* hybridization assay was performed (described below).

Corticosterone radioimmunoassay

Plasma samples (20 μl) were diluted in 0.01 M phosphate buffered saline (PBS; 1 ml) and then heated for 1 h in a 70°C water bath in order to inactivate corticosteroid binding globulin. Plasma samples and corticosterone standards (25–2000 pg/tube) were incubated overnight with rabbit antiserum raised against corticosterone-21-hemisuccinate BSA (B3-163; Endocrine Sciences, Tarzana, CA, USA) and ^3H -corticosterone (Amersham, Bucks, UK, 50 Ci/mmol; 10 000 c.p.m./tube). Antibody-bound steroid was separated from free steroid by mixing with dextran-coated activated charcoal in 0.01 M PBS and centrifugation. Supernatant radioactivity was determined by a liquid scintillation analyser (Packard Instruments, Downers, IL, USA, 1600TR). Intra and interassay coefficients of variability for standards containing 5 $\mu\text{g/dl}$ corticosterone were 14% and 15%, respectively, and for 20 $\mu\text{g/dl}$ corticosterone were 9% and 14%, respectively. The detection limit was 0.5 $\mu\text{g/dl}$ for a 20- μl sample.

ACTH radioimmunoassay

Blood samples for the ACTH assay were collected into EDTA coated tubes and stored at -80°C . Plasma concentrations of ACTH were determined by radioimmunoassay procedures described in Nicholson *et al.* (33) using antiserum (rabbit antibody Rb7) courtesy of Dr Bill Engeland, University of Minnesota. The detection limit for this assay was 15 pg/ml for a 50- μl sample.

Corticosteroid-receptor binding measures

Corticosteroid-receptor binding was performed as described previously (32). Briefly, tissue was homogenized in a buffer solution (10 mM Tris, 1 mM EDTA, 20 mM molybdc acid, 10% glycerol), and centrifuged at 4°C at 20 000 g for 30 min. The supernatant fraction (cytosol) was incubated overnight in the presence of ^3H -dexamethasone (15 nM) with or without nonradioactive competitors. Bound ^3H -dexamethasone was separated from unbound steroid using 1 ml Sephadex (LH-20) columns. The macromolecular fraction was mixed with scintillation cocktail and the radioactivity was measured using a scintillation counter. Available GR was determined from the amount of total ^3H -dexamethasone binding that was displaced by the selective GR agonist RU28362 (0.5 μM). Available MR was determined by the amount of residual ^3H -dexamethasone specific binding. Non-specific binding was defined as the amount of ^3H -dexamethasone binding that was not displaced by an excess of dexamethasone (10 μM). Protein concentrations for each sample were determined using a Bradford assay and included in the determination of receptor levels to control for varying amounts of sample.

Fos protein immunohistochemistry

Tissue sections were incubated overnight (4°C) in PBS containing rabbit antiserum raised against Fos (Santa Cruz Biotechnology, Santa Cruz, CA, USA, #52, 1 : 7500), 0.3% Triton-X and 1.5% normal goat serum. Tissue was then incubated in PBS containing biotinylated goat anti-rabbit Ig secondary antibody (Vectastain, BA-1000; Vector Laboratories, Burlingame, CA, USA) for 2 h and colour precipitate was achieved using the biotin-avidin-immunoperoxidase method (ABC VectaStain Kit, Vector Laboratories). Tris-buffered diaminobenzidine (0.15 mg/ml) and nickel ammonium sulphate (1 mg/ml) were used as a chromogen, and Fos was visualized as a black reaction product. Sections were mounted onto glass microscope slides, air-dried, rinsed in distilled water, dehydrated, delipidized, and coverslipped.

Quantification of Fos-positive nuclei was achieved from coded digitized images of tissue sections using NIH Image software. Nuclei were considered Fos positive if they were stained darker than a cell determined to be of threshold value. This determination was at the discretion of the quantifier and remained consistent across all sections. All sections from a given cohort of rats were processed simultaneously

in a single assay using a common pool of reagents. The absolute cell densities for a given treatment group were similar across assays and therefore were pooled.

In situ hybridization

Before application of radioactive probe, sliced and mounted tissue was fixed in a buffered 4% paraformaldehyde solution for 1 h at room temperature. Slides were washed in $2 \times$ standard sodium citrate (SSC) and acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min to minimize nonspecific hybridization by reducing positive charge on tissue and polylysine coated slides. Slides were then washed again in distilled water, dehydrated in a series of graded ethyl alcohol concentrations, and air dried. ^{35}S -UTP or ^{35}S -UTP/ ^{35}S -CTP labelled cRNA probes were generated for *c-fos* mRNA or CRH hnRNA from cDNA subclones in transcription vectors using standard *in vitro* transcription methodology (24). CRH hnRNA cDNA was kindly provided by Dr Robert Thompson (University of Michigan) and *c-fos* mRNA cDNA was kindly provided by Dr Tom Curran (St Jude Children's Research Hospital, Memphis, TN, USA).

Riboprobes were diluted in hybridization buffer (24) to a concentration of approximately 1–2 million counts per slide (65 μl /slide). Diluted probe was applied and a coverslip was placed on each slide. Slides were placed in sealed plastic boxes lined with chromatography paper moistened with 50% formamide in ddH₂O and were incubated overnight at 55 °C. The next day, coverslips were floated off and slides were rinsed several times in $2 \times$ SSC. The slides were then incubated in RNaseA (Sigma, St Louis, MO, USA) at 37 °C for 1 h to remove unhybridized RNA fragments. Slides were then washed successively in decreasing concentrations of SSC, incubated in a stringent wash of $0.1 \times$ SSC at 70 °C for 1 h to denature weakly bound hybrids, and then dehydrated in graded concentrations of ETOH. Once the slides were dry, they were exposed to Kodak XAR X-ray film (Kodak, Rochester, NY, USA).

Image analysis

Semi-quantitative analyses were performed on digitized images from X-ray films (NIH Image, macro; <http://rsb.info.nih.gov/nih-image/>). Signal pixels of the brain area of interest were defined as being 3.5 SDs above the mean of a cell poor area close to the region of interest representative of background. The number of pixels and the average pixel values above the set background were then computed and multiplied, giving an integrated densitometric measure of arbitrary units (integrated density).

Statistical analysis

Statistical analyses were conducted using the StatView statistical analysis program. Unless otherwise noted, data were analysed using one or two-way analyses of variance (ANOVA) followed by Fisher's post-hoc tests. Error bars in the figures represent \pm SEM.

Results

Ability of peripherally administered RU28362 to occupy corticosteroid receptors in brain

Treatment with RU28362 effectively and selectively occupied GR in both brain areas measured. Figure 2(A,B) shows the effect of RU28362 on available cytosolic GR and MR radioligand binding, respectively, in the hypothalamus and hippocampus.

In the hippocampus, RU28362 caused an almost complete reduction in available cytosolic GR binding [$F(1,11) = 291.4$, $P < 0.0001$] and a small but significant increase in MR binding [$F(1,11) = 5.3$, $P = 0.04$]. RU28362 treatment also significantly reduced available cytosolic GR binding in the hypothalamus [$F(1,9) = 31.5$, $P = 0.0003$]. MR binding in the hypothalamus was barely detectable, but was not significantly altered by RU28362 treatment.

Effect of RU28362 pretreatment on plasma corticosterone concentration and Fos protein expression following restraint stress

Following pretreatment with RU28362, there was a dissociation found between neuronal activation, as measured using Fos

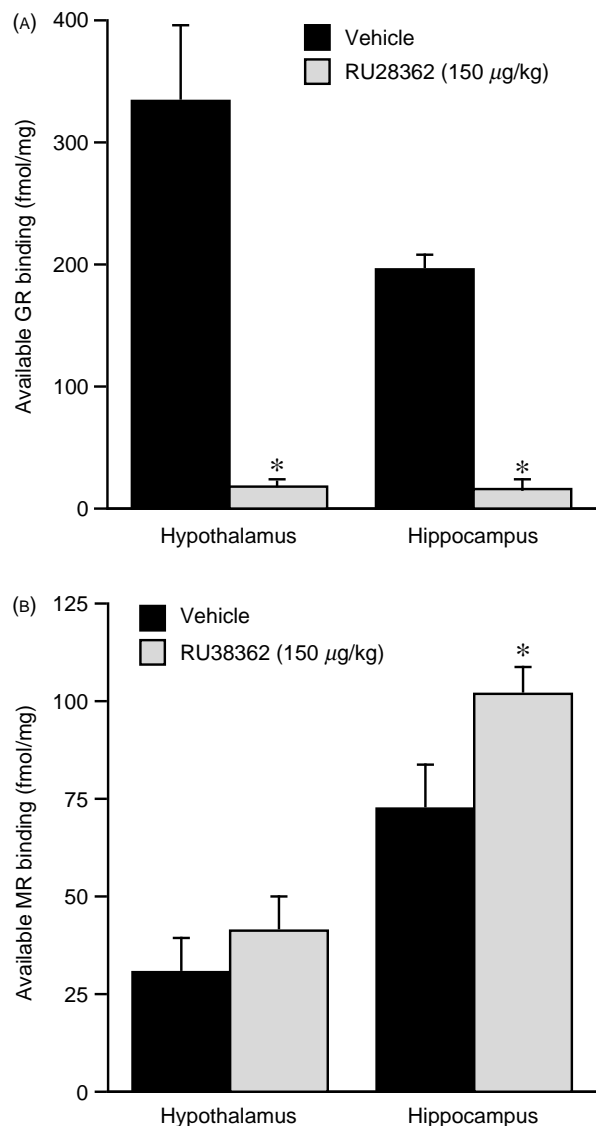


FIG. 2. RU28362 (150 $\mu\text{g}/\text{kg}$) selectively occupied glucocorticoid receptors (GR) in both the hypothalamus and hippocampus one hour following i.p. injection. GR (A) and mineralocorticoid receptors (MR) (B) radioligand binding were measured in brain tissue removed from 24 h adrenalectomized rats. * $P < 0.05$, compared to vehicle-treated values for the same tissue ($n = 6$).

immunohistochemistry, and HPA axis end-product secretion, plasma corticosterone. Rats pretreated with RU28362 and subsequently restrained had significantly less plasma corticosterone than those that were pretreated with vehicle (Fig. 3A) [Student's *t*-test, $t(54) = -8.7$, $P < 0.0001$]. Rats exposed to restraint stress expressed significantly higher levels of Fos protein in the PVN [$F(1,51) = 31.2$, $P < 0.0001$] compared to homecage controls. Drug treatment had no significant effect on Fos expression in the PVN (Figs 3B and 4).

Effect of corticosterone pretreatment on Fos protein expression following restraint stress

To see how occupation of MR, in addition to GR, might affect Fos expression following restraint stress, an experiment with a paradigm identical to the previous one was carried out using a high

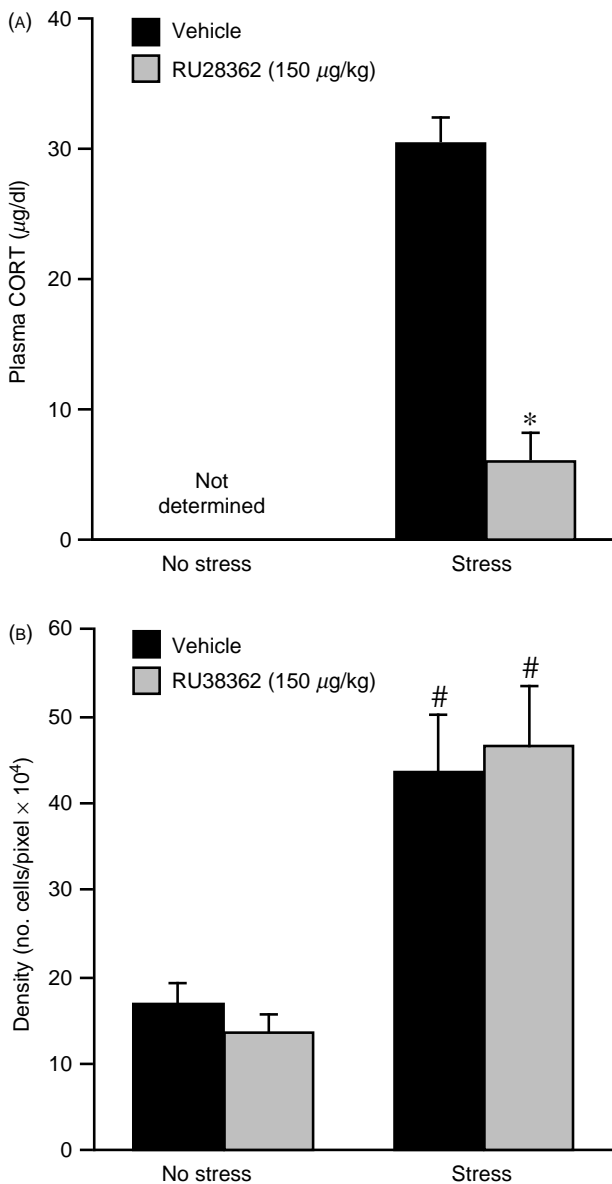


FIG. 3. RU28362 (150 µg/kg) decreased the corticosterone (CORT) response to 1 h restraint stress, but did not block the stress-induced increase in Fos protein expression in the paraventricular nucleus (PVN). Corticosterone (A) was measured in a plasma sample taken 30 min after restraint onset. Fos immunoreactive positive cells (B) were determined in PVN from tissue collected 2 h after onset of 1 h restraint stress. RU28362 or vehicle were injected 1 h before restraint onset. *P < 0.05 compared to vehicle-treated values. #P < 0.05, compared to no-stress values (n = 9–11).

dose corticosterone (5 mg/kg i.p.) pretreatment. Fos expression following corticosterone pretreatment (n = 4) was similar to expression following RU28362 pretreatment. Because exogenous corticosterone treatment invalidates use of plasma corticosterone concentrations as an indication of HPA axis activity, ACTH was measured in this experiment in order to observe HPA activation. Plasma ACTH concentrations in rats exposed to restraint stress were significantly lower following corticosterone pretreatment compared to those pretreated with vehicle alone (Fig. 5A) [Student's *t*-test, $t(16) = -2.075$, $P = 0.05$]. Overall, rats exposed to restraint stress expressed significantly more Fos than homecage controls (Fig. 5B) [$F(1,16) = 51.87$, $P < 0.0001$]. As with

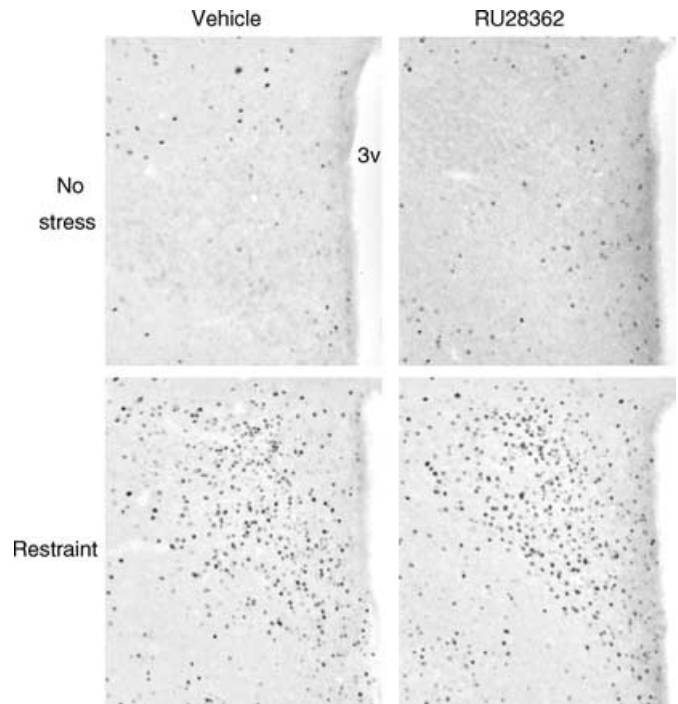


FIG. 4. Representative photomicrographs showing induction of Fos protein in stress-responsive neurones of the paraventricular nucleus 2 h following the onset of 1 h restraint stress. Subjects received either RU28362 (150 µg/kg) or vehicle injection i.p. 1 h before the onset of restraint. Fos protein was visualized by immunohistochemistry; × 20. 3v, Third ventricle.

RU28362 pretreated rats, corticosterone pretreated rats did not express significantly different levels of Fos in the PVN compared to vehicle-pretreated rats.

Effect of RU28362 pretreatment on gene expression following restraint stress

Experiments quantifying *c-fos* mRNA expression following RU28362 pretreatment produced results consistent with those of the protein expression studies. Because each timepoint was collected from a different cohort of animals, two-way ANOVAs were conducted at each timepoint. Plasma corticosterone was significantly reduced by RU28362 pretreatment at both 15 min [$F(1,20) = 26.75$, $P < 0.0001$] and 30 min [$F(1,12) = 76.915$, $P < 0.0001$] (Figs 6A,B) after restraint onset. *c-fos* mRNA was significantly up-regulated in the PVN by stress at both 15 and 30 min [$F(1,12) = 8.95$, $P = 0.01$ and $F(1,17) = 19.152$, $P < 0.001$, respectively] yet was unaffected by drug pretreatment. CRH hnRNA expression was significantly higher following 15 min stress (but not 30 min) compared to homecage controls (Fisher's, $p = 0.01$). By contrast to *c-fos* mRNA, stress-induced CRH hnRNA expression was completely blocked by pretreatment with RU28362 compared to vehicle-treated rats [$F(1,20) = 7.968$, $P = 0.01$] (Figs 7A,B).

Discussion

One hour of restraint caused a substantial increase in plasma corticosterone concentrations, as well as an induction of *c-fos* mRNA, Fos protein, and CRH hnRNA within the PVN. The

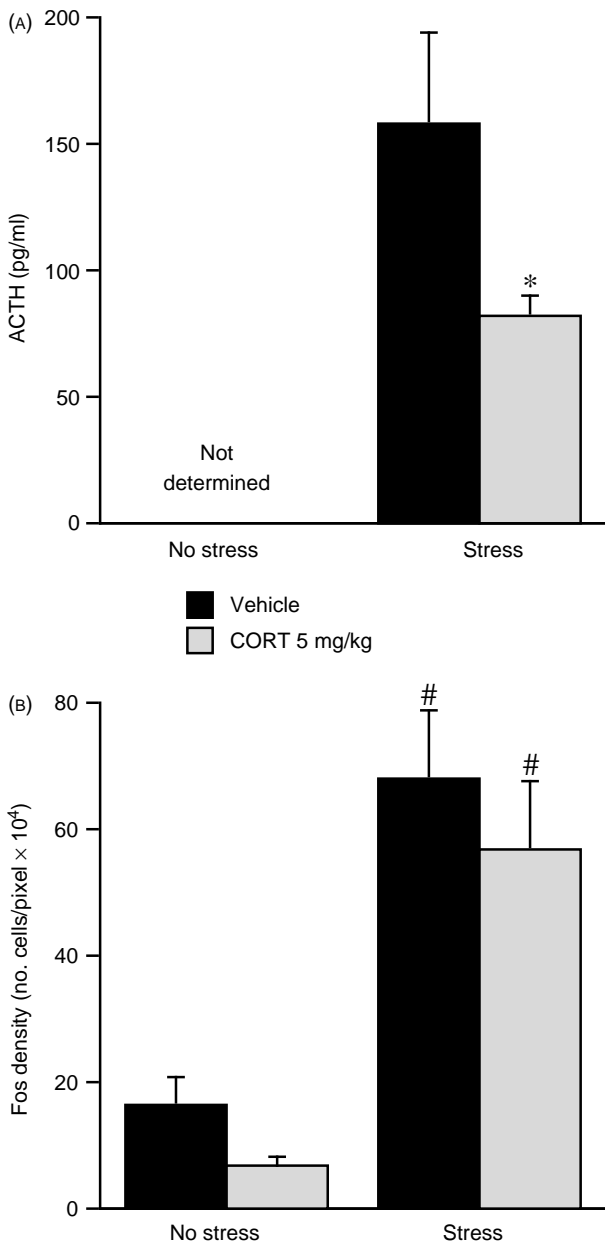


FIG. 5. Corticosterone (CORT) (5 mg/kg) decreased the adrenocorticotrophic hormone (ACTH) response to 1 h restraint stress, but did not block the stress-induced increase in Fos protein expression in the paraventricular nucleus (PVN). ACTH (A) was measured in a plasma sample taken 30 min after restraint onset. Fos immunoreactive positive cells (B) were determined in PVN from tissue collected 2 h after onset of 1 h restraint stress. RU28362 or vehicle were injected 1 h before restraint onset. **P* < 0.05 compared to vehicle-treated values. #*P* < 0.05 compared to no-stress values (*n* = 4).

stress-induced increase in plasma corticosterone and CRH hnRNA expression were almost completely suppressed by acute RU28362 pretreatment. On the other hand, *c-fos* mRNA and Fos protein induction in the PVN were unaffected by GR agonist pretreatment. Similarly, pretreatment with a high dose of corticosterone, which activates both MR and GR, failed to suppress Fos induction. These results indicate that acute GR agonist treatment did not alter the pattern of transynaptic input to the PVN that arises during restraint, at least in as much as that input is transduced into

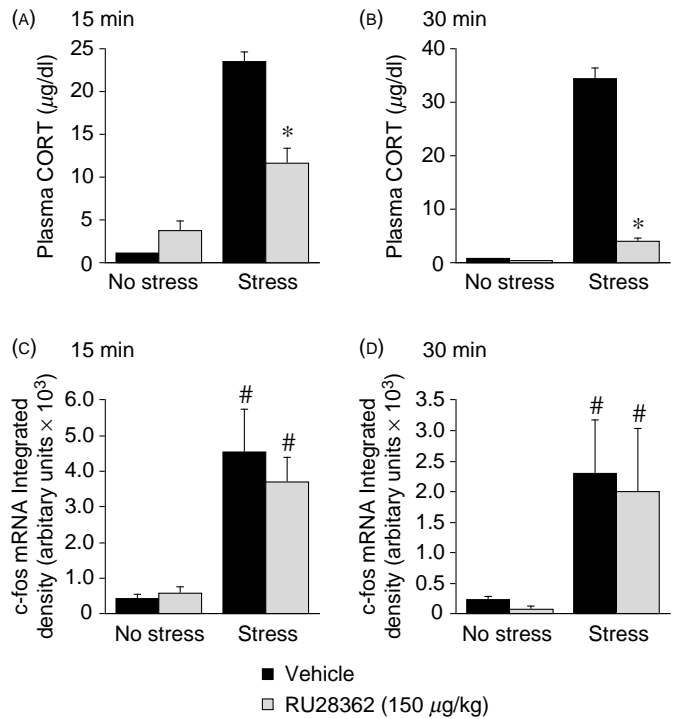


FIG. 6. RU28362 (150 µg/kg) decreased the corticosterone (CORT) response to restraint stress both 15 and 30 min following the onset of restraint, but did not block the stress-induced increase in *c-fos* mRNA in the paraventricular nucleus at either timepoint. Trunk blood corticosterone levels (A,B) or *c-fos* mRNA (C,D) were measured 15 min (A,C; *n* = 6) or 30 min (B,D; *n* = 4) after restraint onset. RU28362 or vehicle were injected 1 h before restraint onset. **P* < 0.05 compared to vehicle-treated values of same stress condition. #*P* < 0.05, compared to no-stress values.

intracellular responses converging on *c-fos* mRNA expression. Nevertheless, this treatment was very effective at suppressing the corticosterone response to restraint.

One possible explanation for the dissociation between *c-fos* expression in the PVN and peripheral indicators of HPA axis activity is that the negative-feedback effects of RU28362 occurred exclusively at the level of the pituitary. In fact, we considered the possibility that this relatively low dose of synthetic glucocorticoid was unable to effectively cross the blood-brain barrier, as has been shown for low doses of dexamethasone, another synthetic glucocorticoid (34). However, our *ex vivo* corticosteroid-receptor binding study showed that the dose of RU28362 used for these studies occupied a majority of GR in the PVN and hippocampus, as determined by a decrease in available cytosolic GR binding after acute RU28362 treatment. In previous studies comparing corticosteroid-receptor binding with a Western blot measure of cytosolic and nuclear GR, we have demonstrated that a decrease in available cytosolic GR binding seen within 1 h after bolus corticosterone treatment is due to GR activation and translocation to the nucleus rather than a rapid down regulation of GR (32). Interestingly, in this study, we saw a small increase in hippocampal MR binding after RU28362 treatment. There is some evidence that GR selective agonists can lead to an increase in MR expression (35), although it is unlikely that such an effect would impact on receptor protein binding within such a short time-frame.

Besides obtaining evidence that GR within the brain were occupied by the dose of RU28362 used in these studies, we also

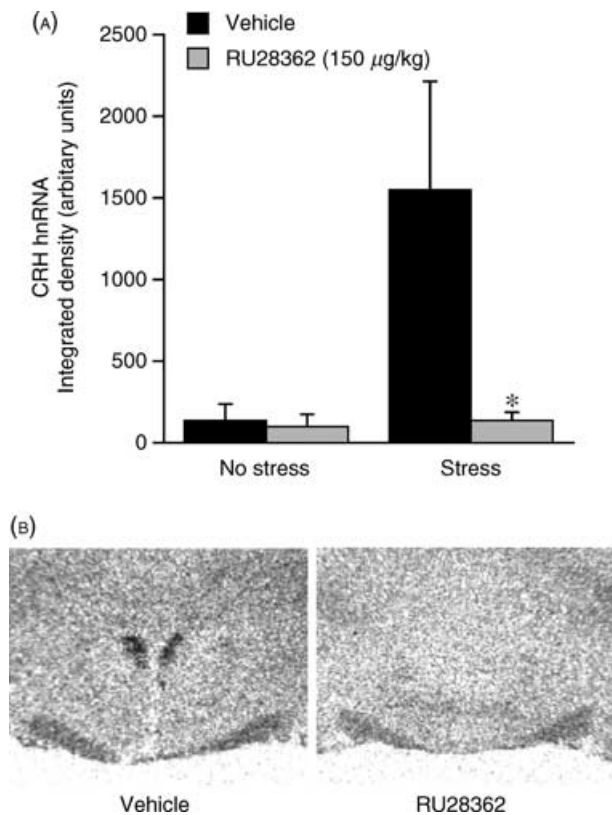


FIG. 7. RU28362 (150 µg/kg) blocked the stress-induced increase in corticotropin-releasing hormone (CRH) hnRNA in the paraventricular nucleus (PVN) (A, * $P < 0.05$). CRH hnRNA (A) was determined in PVN from tissue collected 15 min after restraint onset. RU28362 or vehicle were injected 1 h before restraint onset. * $P < 0.05$ compared to vehicle-treated values of same stress condition ($n = 6$). Representative autoradiograms (B) of stress-induced CRH hnRNA following treatment with RU28362 or vehicle. Expression of CRH hnRNA in the PVN is clearly visible in the vehicle, but not the RU28362 treated rat.

saw that this RU28362 treatment regimen had an effect on CRH neurones in the PVN as evidenced by the inhibition of CRH hnRNA elevation in response to restraint. Thus, within this same population of cells we saw a dissociation between the effects of RU28362 on stress-induced *c-fos* and CRH gene induction. There are other incidences where *c-fos* induction is maintained in the face of suppressed HPA axis hormonal secretion and, conversely, where increased activity of the HPA axis is not associated with increased *c-fos* expression. In the former case, rats with adjuvant arthritis are unable to mount an adrenocorticotrophic response to hypertonic saline injection but express a significantly higher amount of *c-fos* expression in the PVN compared to controls (36). Interestingly, rats with adjuvant arthritis also do not display an increase in CRH mRNA in response to hypertonic saline. Rats in the stress-hyporesponsive phase of development also do not show elevated corticosterone levels in response to a mild stressor, but do show increased *c-fos* expression (37). Conversely, although corticosterone cycles diurnally, *c-fos* expression in the PVN does not, even when corticosterone concentrations at the circadian peak are comparable to those of a substantial stress response in the circadian trough (38).

A number of studies have examined the effects of manipulating glucocorticoid levels in rats on basal and/or stress-induced *c-fos* and CRH gene expression in the PVN. The majority of these

studies have examined only the effects of long-term tonic changes in glucocorticoid levels, resulting from adrenalectomy and chronic replacement with various doses of glucocorticoids. In many cases, although not all, there is evidence for glucocorticoid suppression of basal and stress-induced levels of *c-fos* gene expression in the PVN (29, 39–44). Similar evidence is more consistently found in the case of glucocorticoid regulation of CRH gene expression (29, 36, 44–52). Thus, in many cases there appears to be a tonic inhibitory effect of glucocorticoids upon basal or stress-induced *c-fos* and CRH gene expression in the PVN. Whether this regulation reflects a direct effect of glucocorticoids on gene expression or an indirect effect on excitatory activity of these neurones remains to be determined.

Only a few studies, besides ours, have examined the effects of acute glucocorticoid manipulations on *c-fos* and CRH gene expression. A single injection of rats with metyrapone, a corticosteroid synthesis inhibitor, resulted 60 min later in elevated *c-fos* mRNA and CRH hnRNA within the PVN (30). This response may be a result of an abrupt reduction of basal corticosterone concentrations, or the lack of a negative-feedback effect of glucocorticoids that would normally be secreted in response to the stress of injection. There is also some evidence that metyrapone treatment itself triggers a stress-like response in the brain (53). More directly relevant to our study, several groups have examined the effect of elevating glucocorticoid levels on stress-induced *c-fos* and CRH gene expression. Imaki *et al.* (29) injected rats with a single high dose of the synthetic glucocorticoid dexamethasone 1 h before challenge with 30 min of restraint. Rats with acute dexamethasone treatment had lower *c-fos* mRNA levels than vehicle-treated rats both in the absence of restraint and after restraint, whereas CRH hnRNA was reduced in dexamethasone treated rats only in the postrestraint condition. While the inhibitory effect of acute dexamethasone treatment on stress-induced CRH gene expression is similar to our results, the apparent inhibitory effect on stress-induced *c-fos* mRNA expression is not. Because we failed to see an effect of RU28362 or corticosterone treatment on Fos protein expression 2 h after stress onset, or on *c-fos* mRNA levels 15 and 30 min after restraint onset, the experimental parameter (other than potential pharmacodynamic differences between dexamethasone and RU28362) that explains the difference between our results and that of Imaki *et al.* (29) is not immediately obvious. Consistent with our Fos data, however, two other groups (54, 55) found that injection of rats with a high dose of dexamethasone had no effect on stress-induced Fos protein expression within the PVN. One of these groups (55) also measured CRH hnRNA expression in their study, and, contrary to our results, found no inhibitory effect of dexamethasone. In that study, dexamethasone was administered 10 min before ether stress. No data are shown for rats given a vehicle injection combined with ether stress, so it is possible that the combined stress of injection followed shortly after by ether could have resulted in a greater induction of CRH hnRNA in vehicle pretreated rats than in dexamethasone pretreated rats. It is also possible, as discussed below, that 10 min of dexamethasone pretreatment was not sufficient to fully activate GR before the onset of stress-induced CRH gene induction, especially considering the limited ability of dexamethasone to cross the blood–brain barrier.

The inhibitory effect of RU28362 on stress-induced CRH hnRNA that we observed probably points to an important temporal relationship between glucocorticoid elevation and stress

onset that is necessary for this inhibition to take place. Moreover, the temporal requirements for this effect may partly explain the apparent differential sensitivity to glucocorticoids that has been noted between vasopressin and CRH gene induction in response to acute stress. Ma and Aguilera (56) determined that the rapid induction of CRH hnRNA resulting from the stress of an intraperitoneal injection in adrenalectomized rats was no different in rats injected with a high dose of corticosterone or vehicle. By contrast, they found that the corticosterone injection dramatically reduced the slower to develop vasopressin hnRNA induction compared to the saline injection response. A perhaps important distinction between their experimental conditions and ours was that the elevation of corticosterone was concurrent with the onset of their stress event (injection stress), whereas in our study glucocorticoid treatment occurred 1 h before restraint stress. Kovacs *et al.* (44) found that adrenalectomy had no effect on the kinetics of the CRH hnRNA response to ether stress while significantly advancing the time of the vasopressin hnRNA peak response compared to adrenal-intact rats. These and other results, have led to the conclusion that stress-induced vasopressin gene expression is more sensitive to the negative feedback effects of glucocorticoids than is stress-induced CRH gene expression (56–58). In our study, we find that the rapid induction of CRH gene expression to stress can be dramatically suppressed by the acute presence of glucocorticoids, but it appears that an increase in GR activation must take place before the onset of stress. This is probably not surprising, given the rapid response of CRH gene expression to stress onset. Thus, the elevation in endogenous glucocorticoids that occurs during a single stress episode may not occur soon enough to significantly impact on the CRH gene induction arising from that particular event.

Although RU28362 treatment may act very rapidly to inhibit stress-induced CRH gene expression, such an effect at the neuropeptide transcription level is not likely to account for the suppression of corticosterone secretion that we saw 30 min after restraint onset. Thus, there must be some other negative-feedback mechanism operational within the time-frame of our experiment. In another study, RU28362 injected intravenously produced fast inhibitory effects on CRH-induced ACTH secretion from the pituitary within 5 min (15). Within this time-frame, it is most likely that RU28362 operates by suppressing excitation–secretion coupling of CRH and/or ACTH producing cells rather than via suppressed synthesis of CRH or ACTH (59). Studies using cultured pituitary corticotrophs, AtT20 cells, have shown that intracellular GR can attenuate the CRH-dependent release of ACTH by interfering with protein kinase A mediated inhibition of calcium-activated potassium channels (59, 60). Although this effect depends upon *de novo* protein synthesis, it is relatively rapid (less than 2 h). If hormone activated GR can alter release of ACTH from corticotrophs, that function could be extended to release of CRH into the median eminence from neurones of the PVN. Thus, acute injection of RU28362 may produce neurochemical changes at the nerve terminal to inhibit CRH release without affecting neuronal activation and *c-fos* expression. Whether or not the decrease in stress-induced CRH hnRNA expression after acute glucocorticoid agonist pretreatment would also significantly impact on subsequent CRH peptide stores and HPA axis hormonal secretion is yet to be determined.

These results support the notion that stress-induced signalling and circulating glucocorticoid concentrations are at least partly

integrated at the level of the hypothalamic CRH neurone. However, stressor specificity may be a factor in central responsiveness to glucocorticoid treatment, as various stressors activate the HPA axis via different neural pathways to the PVN that may be glucocorticoid sensitive or insensitive. Moreover, while glucocorticoids may affect input to the PVN, they may simultaneously be exerting direct inhibitory effects at the level of the CRH neurone (CRH synthesis and/or release).

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