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# Time Dependent Effects of Glucocorticoids on Adrenocorticotropin Secretion of Rat Pituitaries Ex-vivo

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**Abstract**

Different glucocorticoids have been compared with respect to the inhibition of corticotropin-releasing factor (CRF)-mediated adrenocorticotropin (ACTH) secretion from pituitary fragments of the rat. The influence of time of exposure to glucocorticoids and glucocorticoid concentration has been investigated.

CRF-stimulated ACTH secretion of perifused rat pituitary fragments was measured by a chemiluminescence immunoassay. ACTH secretion was monitored over three days. Inhibition of CRF-stimulated ACTH secretion by glucocorticoids was quantified by the area under the curve of CRF-stimulated ACTH secretion over baseline. Concentrations needed to inhibit ACTH secretion decreased with the receptor affinities of the glucocorticoids as follows: fluticasone propionate; receptor affinity 1800, concentration $10^{-8}$ M; budesonide, 935 and $3-2{\cdot}5 \times 10^{-8}$ M; flunisolide, 478 and $5 \times 10^{-7}$ M; prednisolone, 10 and $10^{-6}$ M. CRF-stimulated secretion was inhibited by glucocorticoids after incubation for 1 min at concentrations between $10^{-8}$ and $10^{-6}$ M. The same absolute quantity of the glucocorticoids produced no inhibition when incubation was prolonged to 50 min or when a lower concentration was used. Immediately after the perifusion stimulation of ACTH secretion was observed.

The results suggest the possibility of minimizing the side effects of glucocorticoids by prolonging drug release.

One of the most important unwanted effects of glucocorticoids is suppression of the hypothalamic–pituitary–adrenal (HPA) axis. In the case of asthma therapy the effect on the HPA axis could be greatly reduced by inhalation compared with systemic application because targeting of drug to the lung reduces the amount of circulating glucocorticoid considerably. However, glucocorticoid absorbed from lung and the circulating fraction of the swallowed amount may lead dose-dependently to HPA suppression.

Time of administration, plasma level and the affinity of the glucocorticoid to its receptor are factors that determine the extent and duration of HPA axis suppression. Thus, we were interested to investigate the following points: is the suppression of adrenocorticotropin (ACTH) release, stimulated by the corticotropin-releasing factor (CRF), related to the affinities of glucocorticoids to their receptor? Is CRF-stimulated ACTH release influenced by short pulses of glucocorticoids in the same way as by the presence of the same total amount of steroid given over a longer period of time as a diluted solution? Or, more precisely, is the reaction of the pituitary exactly determined by the product of concentration and time of incubation of the glucocorticoids?

The background for that question is the fact that highly lipophilic glucocorticoids are absorbed from the lung slowly so that plasma concentrations are low but half-life in plasma is long. In contrast, glucocorticoids that are better water-soluble are absorbed very rapidly from the lung showing high peak plasma concentrations but a short half-life in plasma (Meibohm et al 1998). Therefore, hypothalamus and pituitary are exposed to rather different concentration gradients of glucocorticoid. The extreme scenarios are a short pulse with a concentrated glucocorticoid solution of the better-soluble steroid, and a perfusion over a longer period of time with a diluted solution of the less-soluble glucocorticoid.

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For our investigations we have used rat pituitary fragments. It has been shown that the glucocorticoid receptor of rats binds different synthetic glucocorticoids in the same rank order as the human glucocorticoid receptor (Rohdewald et al 1985).

Glucocorticoids inhibit CRF-induced ACTH secretion, but not basal ACTH secretion of pituitary fragments (Wildmaier & Dallman 1984). The inhibition was observed only when the glucocorticoid was given before CRF (Shipston & Antoni 1992) or simultaneously with CRF (Wildmaier & Dallman 1984). Familary & Funder (1989) could not demonstrate an inhibition using the same model.

Although it is known that the inhibition of CRF-stimulated ACTH-secretion is dose- and time-dependent (Abou Samra et al 1986), to our knowledge no experiments have been made with exposition times as short as 1 min. A perifusion system using a small flow-through cell and a three way-valve enabled us to perform experiments with rather short perifusion periods. We used the glucocorticoids fluticasone propionate, budesonide and flunisolide because they are widely used as inhaled steroids. Prednisolone was included in the investigation as a systemically applied steroid with a relatively low receptor affinity.

## Materials and Methods

*The perifusion system*

A flow cell constructed from a 2-mL plastic syringe was fed with a peristaltic pump using a flow of $6\,mL\,h^{-1}$. The volume of the perifusion cell was adjusted to 1 mL, the inlet and outlet were covered with nylon net. The whole perifusion system was sterile and held at 37°C. For CRF-stimulation and corticoid application a three-way valve was used. The medium reservoir was gassed with carbogen. All solutions were made in Dulbecco's Modified Eagle Medium (DMEM), containing $4{\cdot}5\,g\,L^{-1}$ glucose, without sodium pyruvate. Fractions of the perifusion medium of 1 mL were collected under cooling to −20°C.

*Pituitary fragments*

Male Wistar rats, 250–300 mg, were held for four days under controlled conditions in the research unit at the University to acclimatize them to the new environment. The animals were decapitated, nearly free of stress, by using a Harward animal-decapitator. To keep the influence of the diurnal rhythm of ACTH secretion constant, the animals were killed between 0930 and 1030 h in each series of experiments. The pituitary gland was removed 5–10 min later under aseptic conditions and cut into fragments of approximately $1\,mm^3$. The bottom of the perifusion cells was covered with 200 $\mu$L Sephadex G-10 to reduce the shearing stress during perifusion. Fragments were placed on the Sephadex bed and immediately perifused.

*CRF stimulation*

CRF (Sigma, Germany) was dissolved in DMEM. Solutions containing 100 nM CRF were stored as samples in liquid nitrogen until usage. A reproducible reaction of ACTH secretion to CRF-stimulation could not be achieved on the first day of perifusion, therefore experiments were made on the second and third day. On the fourth day of perifusion no stimulation of ACTH secretion by CRF was observed.

*Glucocorticoid solutions*

Budesonide (AB Draco, Sweden), flunisolide (Krewel-Werke, Germany), fluticasone propionate (Glaxo-Wellcome, UK) and prednisolone (Hoechst AG, Germany) were dissolved in ethanol and diluted with DMEM to the final concentrations. Ethanol concentration in the final solutions did not exceed 0·1%.

*ACTH assay*

ACTH was determined by a chemiluminescence assay from Nichols Institute Diagnostics. The assay was developed to quantify human ACTH but since ACTH from rat differs from human ACTH only in two of the 39 amino acids, the test could be used for the determination of ACTH from rats. Calibration curves made with rat ACTH (Sigma, Germany) showed identical results compared with human ACTH (standard, Nichols Institute Diagnostics) between 0·1 and $1\,ng\,mL^{-1}$. A higher light emission was observed for rat ACTH between $5–100\,pg\,mL^{-1}$, however, sensitivity was lowered. Recovery and reproducibility were tested with three concentrations (n = 6): $26\,pg\,mL^{-1}$ 99·5 ± 5%, $306\,pg\,mL^{-1}$ 97·9 ± 3·1%, $555\,pg\,mL^{-1}$ 99·8 ± 3·1%. Standard curves were obtained by dissolving the rat ACTH in the perifusion medium (DMEM).

Correlation between receptor affinities of the glucocorticoids and inhibition of ACTH-release was calculated according to the following equation:

$$f(x) = 1{\cdot}82 \times 10^3 \times \exp(-4{\cdot}71 \times 10^6 \times x)$$

The coefficient of the exponential correlation was 0·949.

## Results

Flow rates increasing from 6 to 9 and 12 mL $h^{-1}$ produced higher basal secretion of ACTH; however, the response to CRF stimulation was reduced (data not shown). Therefore, perifusion experiments were performed with a flow rate of 6 mL $h^{-1}$.

Repeated stimulation with $10^{-7}$ M CRF for 10 min was performed on two subsequent days to investigate the influence of the glucocorticoid solvent (DMEM + 0·1% ethanol) on ACTH secretion under the conditions of the experiments with glucocorticoid addition. Basal ACTH secretion or the response to CRF stimulation was not influenced by the addition of the solvent for 1 or 50 min (Figure 1A and B). The experiments demonstrate a stable baseline of ACTH secretion and a reproducible response to CRF stimulation over a period of 8–9 h on two subsequent days. The areas under the curve (AUC) of stimulated CRF response over baseline were nearly constant in both cases, expressed as percentage of the AUC before addition of the solvent. This small variation of the AUC is given in Figures 2–6 as a shaded area.

*Fluticasone propionate*

The exposure of the pituitary fragments to $10^{-8}$ M fluticasone propionate for 1 min immediately before stimulation with CRF reduced considerably the AUC of the stimulated CRF response in two experiments (Figure 2). The same absolute amount of fluticasone propionate given over a 50-min period as $2 \times 10^{-10}$ M solution produced, with the exception of one point, no inhibition. In contrast, CRF-stimulated ACTH secretion increased after addition of the glucocorticoid solution relative to the control (Figure 2). Incubation for 30 s with $2 \times 10^{-8}$ M fluticasone propionate produced an inhibition of CRF response, however, the decrease was less pronounced compared with the effect of 1-min exposure to $10^{-8}$ M (Figure 2).

*Budesonide*

Budesonide has a threefold lower clinical efficacy than fluticasone propionate (Rohdewald 1998), therefore its influence was tested at $3 \times 10^{-8}$ M for 1 min (Figure 3). Compared with control, the first stimulation after the addition of the glucocorticoid gave a higher peak of ACTH concentration, thereafter, reaction to CRF was inhibited over the 5-h observation period. The same absolute amount of budesonide applied over 50 min produced a higher ACTH secretion immediately after glucocorticoid perifusion (Figure 3), a slight inhibition relative to control was seen only after 5 h.

Due to the inconstant baseline in the experiment with $3 \times 10^{-8}$ M budesonide another experiment

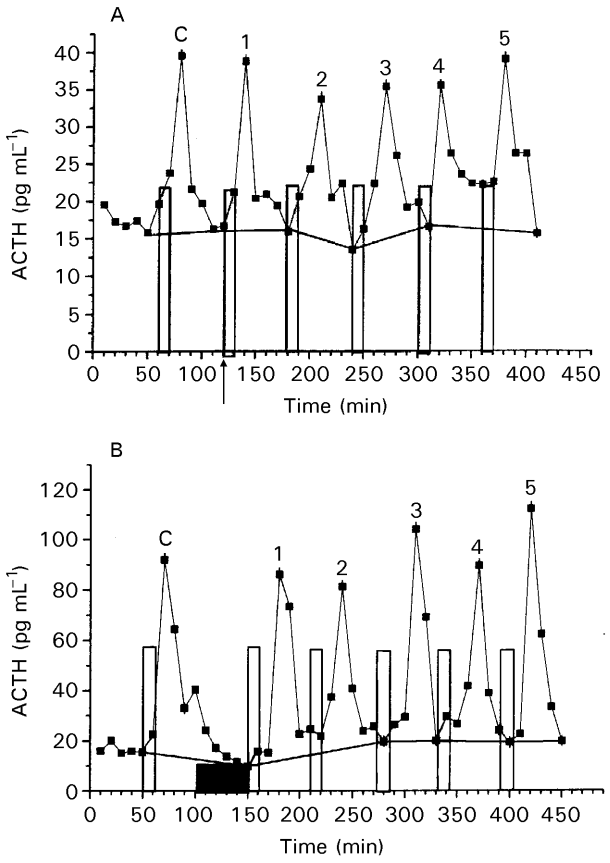


Figure 1. ACTH secretion of perifused rat pituitary fragments. C, control stimulation with CRF; 1–5, subsequent stimulations with CRF following the exposure to solvent (DMEM + 0·1% ethanol) for 1 min (A) or for 50 min (B). The arrow indicates time point and duration of exposure.

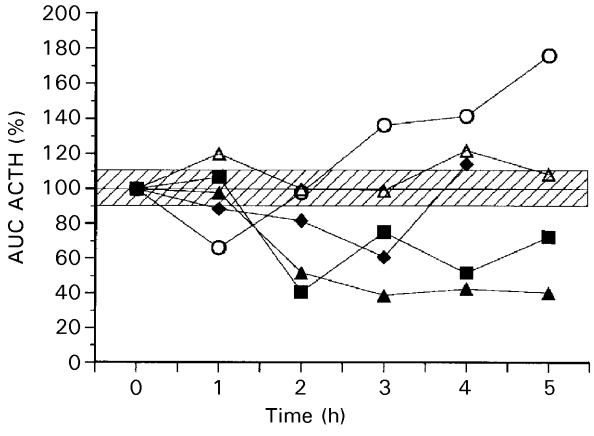


Figure 2. Percentage of the AUCs of CRF-stimulated ACTH secretion over baseline relative to control (100%) following incubation with fluticasone propionate $10^{-8}$ M for 30 s (◆), 1 min (■, ▲), and $2 \times 10^{-10}$ M for 50 min (○, △). Shaded area: variation of AUCs after perifusion with solvent.

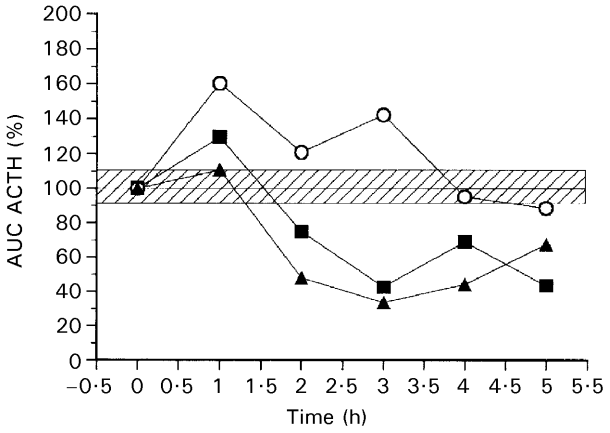


Figure 3. Percentage of the AUCs of CRF-stimulated ACTH secretion over baseline relative to control (100%) following incubation with budesonide $2{\cdot}5 \times 10^{-8}$ M (▲) and $3 \times 10^{-8}$ M (■) for 1 min and with $6 \times 10^{-10}$ M for 50 min (○).

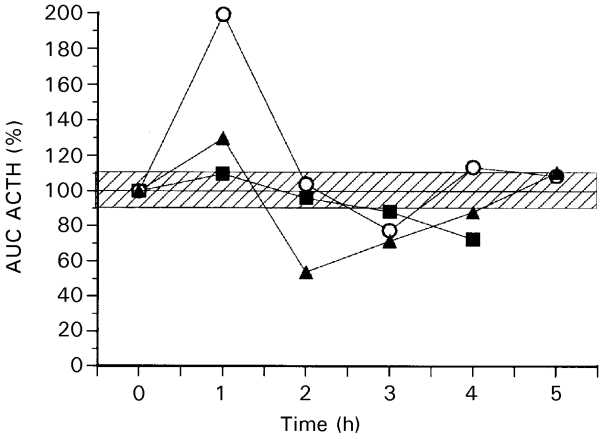


Figure 4. Percentage of the AUCs of CRF-stimulated ACTH secretion over baseline relative to control (100%) following incubation with flunisolide $5 \times 10^{-7}$ M (▲) and $10^{-7}$ M (■) for 1 min and with $2 \times 10^{-9}$ M for 50 min (○).

was performed with $2{\cdot}5 \times 10^{-8}$ M budesonide for 1 min (Figure 3). In that experiment the first peak after budesonide perifusion was greater, and the following stimulations with CRF gave a reduced output of ACTH.

*Flunisolide*

Flunisolide has about half the affinity of budesonide to the glucocorticoid receptor (relative affinity 935 for budesonide, 478 for flunisolide) relative to the affinity of dexamethasone as 100 (Würthwein et al 1992). Therefore, higher concentrations of flunisolide were used compared with the series made with budesonide. Perifusion with different concentrations showed pronounced inhibition for $10^{-6}$ M (data not shown) and a very small inhibition after 1 min with $10^{-7}$ M flunisolide (Figure 4). Perifusion with $5 \times 10^{-7}$ M flunisolide for 1 min yielded an increased ACTH secretion immediately after addition of flunisolide, thereafter, a partial inhibition over 3 h and finally a normal response after 4 h (Figure 4). Perifusion with $2 \times 10^{-9}$ M flunisolide for 50 min caused a doubled response to CRF immediately following flunisolide application, all other values were higher than control, and a reduced response was only noted after 3 h.

*Prednisolone*

Prednisolone has a relative receptor affinity of 10 (Würthwein et al 1992). Therefore, a 100-fold concentration compared with the series with fluticasone propionate (relative receptor affinity 1800) (Würthwein et al 1992) was chosen. Exposure for 1 min with $10^{-6}$ M prednisolone evoked an increase of ACTH response after the first CRF stimulation, subsequently secretion was reduced with a minimum after 4 h (Figure 5). The same total amount given over 50 min ($2 \times 10^{-8}$ M) gave a higher ACTH peak after the incubation period, however, values decreased after the third stimulation showing a slight inhibition of ACTH secretion (Figure 5).

The results can be summarized as follows, using the means and standard deviations of the experiments. The short time incubation with glucocorticoids in concentrations selected according to their relative affinities to the glucocorticoid receptor enhanced the effect of the first CRF stimulation and inhibited ACTH secretion partly during the following stimulations. A tendency to higher values is seen after 5 h (Figure 6). The same amount of glucocorticoid, perifused for 50 min, evoked a higher ACTH secretion after the first stimulation, but the following stimulations produced a nearly normal ACTH response (Figure 6).

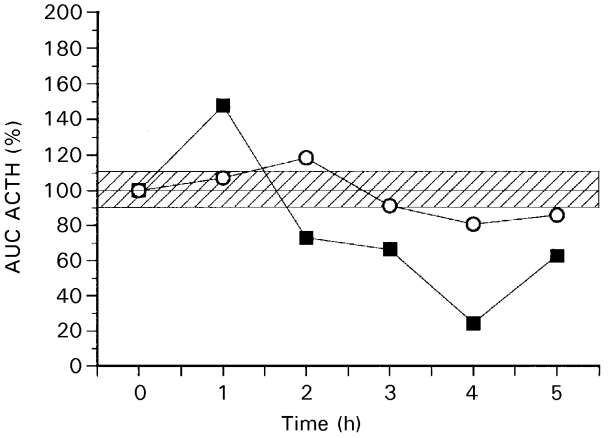


Figure 5. Percentage of the AUCs of CRF-stimulated ACTH secretion over baseline relative to control (100%) following incubation with prednisolone $10^{-6}$ for 1 min (■) and with $2 \times 10^{-8}$ M for 50 min (○).

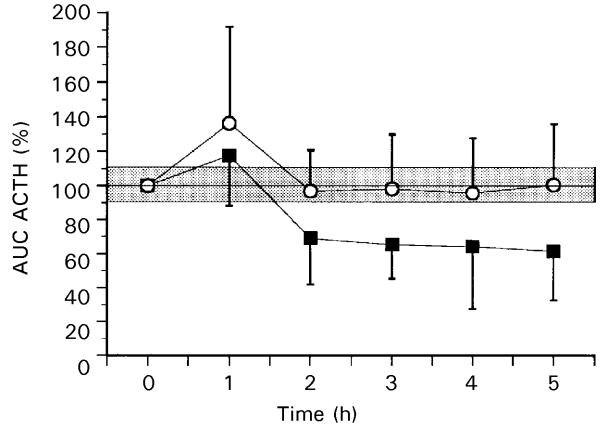


Figure 6. Mean percentage and standard deviation of the AUCs of CRF-stimulated ACTH secretion over baseline relative to control (100%) following incubation with inhibiting concentrations of the different glucocorticoids for 1 min (■) (n = 11) and for the 50-times lower concentrations applied over a period of 50 min (○) (n = 8).

The concentrations of the different glucocorticoids needed to inhibit CRF-stimulated ACTH release after perifusion of pituitaries for 1 min showed a good correlation with the relative receptor affinities (Figure 7).

## Discussion

Our experiments demonstrated that perifused pituitary fragments responded to CRF stimulation in a reproducible way even after three days. However, to obtain an ACTH secretion for evaluation on the second and third day it was necessary to perform five CRF stimulations on the first day after the isolation of the pituitary fragments. Even when the ACTH secretion on the first day was not stable, the stimulation was needed to obtain a sufficiently high basal ACTH secretion and a response to CRF on the following days. Experiments made on the second day without the preceding stimulation gave a baseline secretion below 5 pg $mL^{-1}$ and essentially no response to CRF (data not shown).

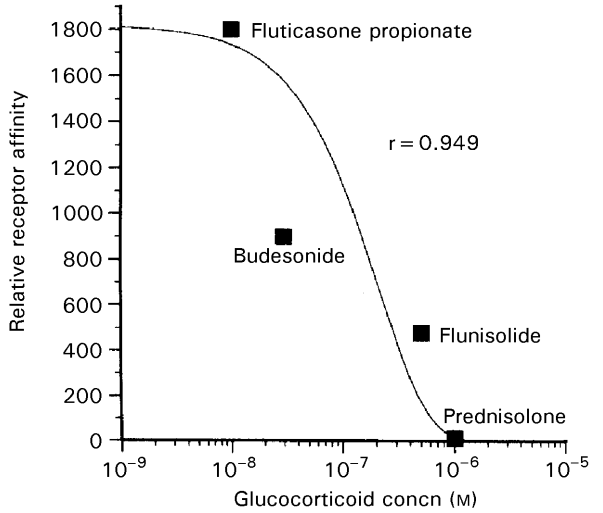


Figure 7. Correlation of inhibitory concentrations of glucocorticoids with their relative receptor affinities.

As expected, baseline ACTH secretion as well as the response to CRF decreased over time. However, during the observation period, baseline secretion was relatively stable in most cases and CRF-response was reproducible within one series of experiments.

Our experiments demonstrated that perifusion of pituitary fragments for 1 min and even for 30 s was sufficient to partly inhibit CRF-stimulated ACTH secretion. Association of glucocorticoids with human glucocorticoid receptor in-vitro was not completed before 30 or 45 min (Högger & Rohdewald 1998). From these experiments, a very limited fraction of receptor-bound glucocorticoids not exceeding 10% could be calculated for an incubation of 1 min.

Therefore, it is remarkable that the glucocorticoids inhibited ACTH secretion after such a short exposition time. However, the strong binding of lipophilic glucocorticoids to tissues may form a deposit-delivering substance for specific binding later on.

In all experiments no inhibition was registered before 2 h, the ACTH release following the stimulation with CRF after 1 h was not inhibited.

Dayanithi & Antoni (1989) reported that the production of mRNA after activation of the glucocortcoid receptor is needed to inhibit ACTH release. That means that glucocorticoids do not simply directly block receptors responsible for ACTH release, but induce a protein blocking the CRF-stimulated ACTH secretion. Therefore, no immediate reaction was expected, nevertheless, the inhibition starting within 2 h indicated a relatively fast regulation of ACTH secretion.

In most experiments the first CRF stimulation following the perifusion with glucocorticoid produced a higher ACTH release, independent from the time of incubation.

An increase of ACTH secretion immediately after glucocortcoid incubation was reported for the AtT20 cell line (Johnson et al 1980). In patients with Morbus Cushing an increase of ACTH secretion was found immediately after cortisol administration (Fehm et al 1977). This paradoxical reaction of ACTH secretion after glucocorticoid incubation of pituitary cells, also observed in our investigation, has not yet been clarified. It can be concluded that a slow release of glucocorticoids

following deposition in the airways could be advantageous. This is because the same total amount of drug, given at a low concentration over a longer period of time, does not inhibit CRF-induced ACTH release, in contrast to a short incubation with a relatively high concentration.

Even though a direct extrapolation from our experiments with rats to man is not possible, we can expect similar conditions for the inhibition of ACTH release. At least the affinities of the synthetic glucocorticoids to the glucocorticoid receptor of the rat follow the same rank order as for the human glucocorticoid receptor (Rohdewald et al 1985). Hence, it is highly probable that the therapeutic index of an inhaled glucocorticoid will be significantly improved if its concentration in lung tissue can be maintained high enough to achieve an anti-inflammatory action and to ensure that release from the tissue is insufficient to influence pituitary function.

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