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Glucocorticoids Rapidly Inhibit Oxytocin-Stimulated Adrenocorticotropin Release from Rat Anterior Pituitary Cells, Without Modifying Intracellular Calcium Transients*

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ABSTRACT

Glucocorticoid hormones suppress the secretion of ACTH evoked by secretagogues such as CRF and arginine vasopressin. In this study, we investigated the effects of glucocorticoids on ACTH release induced by oxytocin (OT) and on intracellular free calcium ion levels in corticotropes prepared from the adenohypophyses of female Wistar rats.

Pulsatile additions of physiological concentration of OT (10 nM) to superfused anterior pituitary cells caused pulsatile ACTH release about 4-fold above basal secretion with similar peak amounts of ACTH during subsequent OT pulses. Exposure of the cells to corticosterone (100 nM) or to a selective glucocorticoid receptor agonist RU 28362 (100 nM) for 30 min suppressed OT-stimulated but not basal ACTH release by approximately 60%. Inhibition gradually disappeared during subsequent pulses of OT in the absence of corticosterone. Pretreatment with the selective antagonist RU 38486 (1 μM) completely blocked the inhibitory effect of corticosterone on OT-induced ACTH secretion. Changes in free cytosolic calcium levels in single cultured pituitary cells were measured using the calcium indicator Fura-2. OT caused calcium transients in corticotropes, which were identified by immunocytochemistry. They responded in a similar manner to a second OT stimulus when preincubated for 30 min with corticosterone (1 μM) or with RU 28362 (1 μM). Our data indicate that glucocorticoids, via glucocorticoid receptors, rapidly inhibit OT-stimulated ACTH secretion by corticotropes without affecting intracellular calcium transients due to OT. Therefore, we conclude that rapid inhibition of ACTH release by glucocorticoids interferes with cellular signal transduction beyond the step of calcium mobilization. (*Endocrinology* **132:** 873–878, 1993)

IT IS well established that the secretion of ACTH by anterior pituitary corticotrope cells is stimulated by hypothalamic neurohormones, such as CRF or arginine vasopressin (AVP) (1), and is suppressed by adrenal glucocorticoid hormones (2). Several lines of evidence support the notion that oxytocin (OT) is involved in the regulation of pituitary ACTH secretion; it is present in high concentrations in rat hypophysial portal blood (3, 4), and high affinity receptors for OT have been identified in the rat pituitary (5). Recently, we have demonstrated that physiological concentrations of OT added in pulsatile fashion to isolated rat pituitary cells stimulate ACTH secretion, independent of other ACTH secretagogues (6). OT may therefore function as a hypophysiotropic hormone contributing to physiological regulation of ACTH release by the hypothalamus. In addition, we showed that OT, like AVP and CRF, causes rises in free cytosolic calcium levels in corticotropes, suggesting that calcium transients play the key role to trigger ACTH secretion (7–9). Indeed, studies with permeabilized cell preparations indicate that calcium is the main trigger substance in controlling exocytosis in exocrine (10, 11) and endocrine cells (12–15), including a corticotropic tumor cell line (16).

Glucocorticoid feedback inhibition of stimulated ACTH secretion comprises several time domains (17). Acute suppression is evident within less than one hour of glucocorticoid administration, but the mechanism of the inhibitory action is unknown. In earlier studies (18–20) it was shown that a brief (20–40 min) exposure to corticosterone strongly inhibited ACTH secretion evoked by CRF or AVP. This inhibitory effect was fully antagonized by the glucocorticoid/progestin-antagonist RU 38486, indicating that it is exerted through glucocorticoid receptors (21).

There have been inconsistent reports using a mouse pituitary tumor cell line (AtT-20) regarding glucocorticoid effects on CRF-induced transients in intracellular calcium. In a recent study (22) it was reported that intracellular calcium signals due to CRF stimulation are suppressed by glucocorticoid hormones. But, using the same model, no change in intracellular calcium transients in the presence of glucocorticoids was recently reported (23). In order to understand the action of glucocorticoids on ACTH release and on intracellular calcium levels within normal pituitary corticotropes, we examined in the present study: 1) whether OT-induced ACTH secretion is inhibited by glucocorticoids; and 2) whether glucocorticoids modify transients in free cytosolic calcium levels triggered by OT.

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Materials and Methods

Perifusion of isolated rat anterior pituitary cells

The experimental procedures employed in this study have previously been described (21, 24) with necessary modifications (6). Briefly, dispersed rat anterior pituitary cells were prepared by tryptic digestion from the adenohypophyses of female Wistar rats (250–350 g body wt; obtained from Charles River Wiga GmbH, Sulzfeld, Germany). The cells were mixed with preswollen Sephadex G-10 (Pharmacia, Uppsala, Sweden) and transferred to perifusion columns (volume 0.4 ml) which were then perifused at 37 C with 22 mM HEPES-buffered (pH 7.4) Dulbecco's minimal essential medium (Flow Laboratories, Irvine, Scotland) containing 0.25% (wt/vol) BSA (fraction V, Miles Laboratories, Elkhart, IN) and antibiotic-antimycotic drugs (Sigma, Deisenhofen, Germany). After an equilibration period of 2 h, various treatments were applied. OT was given every 30 min for a 5-min period. Glucocorticoid receptor agonists (corticosterone, RU 28362) were applied 25 min before the application of the second OT stimulus and were also included in the medium during the second exposure to OT. Glucocorticoid receptor antagonist (RU 38486) was given 35 min before the second OT stimulus and was also present during exposure to corticosterone and OT. The synthetic steroids RU 28362, a selective glucocorticoid receptor agonist (25) and RU 38486, a glucocorticoid/progestin antagonist (26) were generously supplied by Roussel Uclaf (Romainville, France). Steroids were dissolved in dimethylsulfoxide (DMSO; Sigma, Deisenhofen, Germany) and diluted in perifusion medium to the desired dilution. The final concentration of DMSO in the perifusion medium did not exceed 0.001%.

Two-minute fractions of the column effluents were collected during the experimental period and assayed for ACTH content by RIA (27) using antiserum AS 6 (courtesy of G. B. Makara, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary). Purified human $ACTH_{1-39}$ (Sigma) was used as the standard. Data are reported as net ACTH release standardized within each cell column. The reference values taken as 100% are given in the figure legends. Data are expressed as the mean ± SEM. Statistical evaluation was performed by two-way nonparametric analysis of variance, followed by Student's two-tailed unpaired *t* test.

Primary cultures of pituitary cells

Pituitary cells were prepared as described previously (6). Aliquots of cell suspensions were plated in poly-L-lysine-coated plastic petri dishes with Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany) buffered with 26 mM sodium bicarbonate, pH 7.0, containing 20 mM cytosin-β-D-arabino-furanoside and 10% fetal calf serum and incubated in a humidified atmosphere (5% CO_2/95% air) at 37 C for 3–5 days. The medium was changed after 24 h and subsequently every 48 h.

Measurement of intracellular free calcium levels

The procedures used to measure intracellular free calcium levels in single cells have been described (6). Briefly, the culture dish was washed with serum-free medium, and the cells were loaded with Fura-2/AM (Calbiochem, La Jolla, CA; 1.5 μM final concentration, 0.1% DMSO) for 30 min at 37 C. The cells were then washed with modified Locke medium (140 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM ascorbic acid, 11 mM glucose, 15 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), buffered to pH 7.2 with NaOH). The same medium was used for calcium measurements. The cells were then subjected to fluorescence measurements with a Zeiss microscope photometer system (FFP, Zeiss, Oberkochen, Germany) up to 2 h after dye loading. The system is based on an inverted microscope (Axiovert 405M) equipped for epifluorescence, and interference filters of 340/10 nm and 380/10 nm are alternatively mounted on the filter wheel. The ratio calculations r = F340/F380 were carried out according to Grynkiewicz *et al.* (28).

Randomly selected cells from the culture dish were treated with an OT stimulus and recorded for 2 min. Responding cells were video printed. The cells were then washed twice and were incubated with corticosterone (1 μM) or RU 28362 (1 μM) for 25 min. The same cells were then subjected to a second OT stimulus in the presence of the steroid. After the calcium measurements, corticotropes were identified among the mixed cell population in the primary cultures by immunocytochemistry as described below.

Immunocytochemistry of pituitary corticotropes in primary culture

After the calcium measurements, the cells were fixed in cold paraformaldehyde (4% in 10 mM PBS, pH 7.2) for 30 min and incubated with anti-ACTH antiserum (courtesy of K. Kovacs, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary; final dilution 1:2000) overnight at 4 C. Antigen-antibody complexes were visualized by the double antibody avidin-biotin-peroxidase complex technique as described earlier (6). Antiserum specificity and method specificity were performed by omitting the primary antiserum and incubations with respective buffer or incubations with normal rabbit serum (1:2000). A coordinate system within the FFP allowed identification of cells previously analyzed for calcium transients.

Results

ACTH release by OT and inhibition by corticosterone

The kinetics of ACTH release due to OT were first examined with a concentration of the stimulating agent similar to that found in rat portal blood. Figure 1A shows the ACTH release by dispersed perifused rat anterior pituitary cells caused by five subsequent pulses of 10 nM OT. Successive applications of OT every 30 min for a 5-min period released similar amounts of ACTH up to 3 h. During the experimental period, basal ACTH release was approximately 1–2% (15 ± 5 fmol/2-min fraction) of the total release and remained nearly constant throughout the experiment.

Application of corticosterone at 100 nM for 25 min before and 5 min during the second OT stimulus suppressed ACTH release elicited by OT by approximately 60% (Fig. 1B). Basal ACTH release was not affected. The effect of corticosterone within this 30-min time domain was marked and statistically significant ($P < 0.05$). This effect of corticosterone was almost completely reversed within 180 min of subsequent OT addition in the absence of further corticosterone application. Stimulation of ACTH release by higher doses of OT (10 μM) was inhibited by corticosterone (100 nM) to a similar extent as that observed with 10 nM OT. In these experiments, after exposure to corticosterone at 100 nM (similar experimental protocol as in Fig. 1B) the release of ACTH was inhibited by 67% at the second OT stimulus and 48%, 27%, and 21% during subsequent pulses of OT (mean of two experiments). Furthermore, pituitary cells from primary cultures (4 days) were loaded into perifusion columns, and a similar corticosterone treatment as in Fig. 1 was applied. This experimental paradigm is important in order to compare the measurements of changes in intracellular calcium levels from primary cell cultures in the absence or presence of glucocorticoid hormones. Interestingly, we did not observe any difference in the net ACTH response due to OT (data not shown), and the inhibition by 100 nM corticosterone of OT-induced ACTH release was 35% at the second OT stimulus and 32%, 21%, and 7% during subsequent pulses of OT.

Effect of glucocorticoid agonists and antagonists on OT-induced ACTH release

The net ACTH release due to OT in the presence or absence of various glucocorticoids is shown in Figs. 2 and 3.

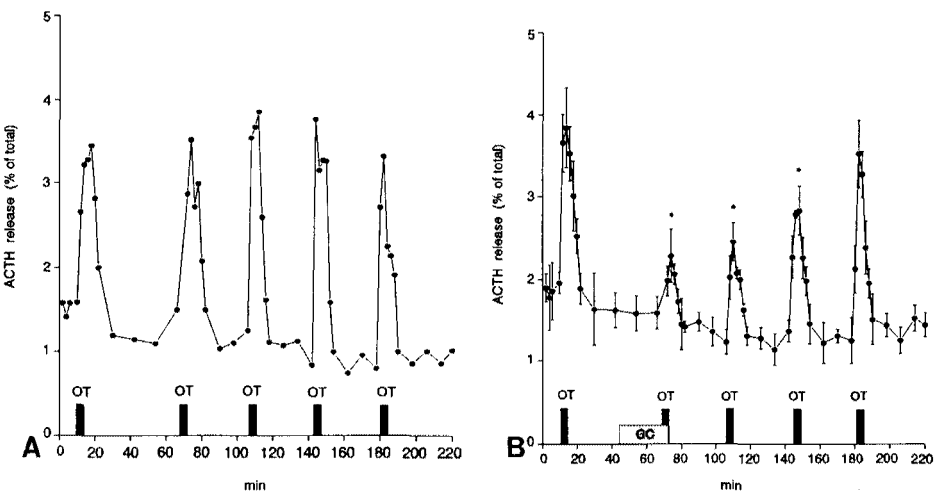

FIG. 1. Release of ACTH by perifused rat anterior pituitary cells in response to subsequent pulses of 10 nM OT (A) and the effect of 100 nM corticosterone on this process (B). Perifused cells were exposed to 100 nM corticosterone for 25 min as indicated by the *bar* (GC); the *black bars* show application of 10 nM OT for 5 min every 30 min. Data are expressed as percentage of total amount of ACTH detected in all fractions assayed. The control experiment (A) represents the values from a single column run at the same time with other columns, where the cells were exposed to steroids simultaneously. The *points* represent means, and the *bars* show SEM; n = 3. The effect of corticosterone (B) was significant ($P < 0.05$) 25 min, 60 min, and 90 min after steroid application.

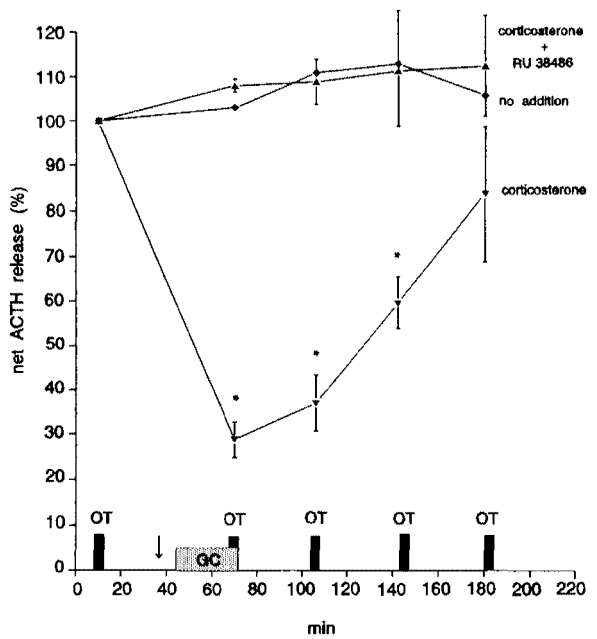

FIG. 2. Effect of 100 nM corticosterone and antagonism by RU 38486 (1 μM) on OT-induced ACTH release. Data are expressed as percentage of the net amount of ACTH release of the first control exposure to 10 nM OT (range, 42–88 fmol/10 min). The *points* are means, and the *bars* show the SEM. RU 38486 was given 10 min before corticosterone application (*arrow*) and was also present during the 25 min of corticosterone application (GC). The *black bars* show application of OT at 10 nM for 5 min. Note that the data of the corticosterone experiment correspond to Fig. 1B. *, $P < 0.05$ when compared to control group (◆).

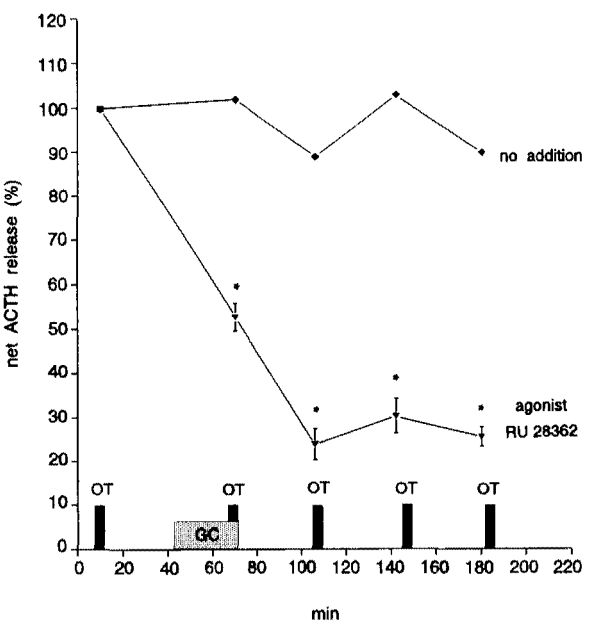

FIG. 3. Effects of glucocorticoid agonist RU 28362 (100 nM) on ACTH release induced by 10 nM OT. RU 28362 (GC) was present 25 min before and 5 min during the second OT stimulus. The 100% value is the net ACTH release in response to the first OT stimulus applied (range, 40–90 fmol/10 min). The control experiment (◆) was run with the same cell preparation. The glucocorticoid agonist effect was significant ($P < 0.05$) at all following OT stimuli. The *points* are the means of three individual experiments; the *bars* show the SEM.

The glucocorticoid/progestin receptor antagonist RU 38486 (1 μM) completely blocked the inhibitory action of corticosterone (Fig. 2). In this experiment, RU 38486 was present 10 min before and also included into the perifusion medium during the 25-min application of corticosterone before the second OT challenge. The corticosterone effect was antagonized throughout the experimental period.

In order to further characterize the glucocorticoid effect on OT-stimulated ACTH release, the cells were exposed for 25 min to the highly selective glucocorticoid receptor agonist RU 28362 at a concentration of 100 nM. RU 28362 potently suppressed OT-induced ACTH release by 80%, and this inhibition persisted for 2 h (Fig. 3).

Transients in intracellular free calcium levels due to OT in the absence or presence of corticosterone or RU 28362

Cytoplasmic calcium levels were analyzed in cultures from several different pituitary cell preparations and more than 200 cells were investigated in this study. They were stimulated individually with 100 nM OT, and intracellular calcium levels were recorded for a 2-min period. The values given below represent data for OT-responsive and identified corticotropes.

In unstimulated corticotropes the basal calcium levels were stable and ranged from 70–100 nM (n = 21 corticotropes out of 225 examined cells). As already observed in our previous study (6), 100 nM OT increased the cytosolic free calcium concentrations in identified corticotropes (Figs. 4A and 5A). Figure 4B shows a representative calcium transient in 1 of 6 immunocytochemically identified corticotropes, which was then incubated for 25 min with 1 μM corticosterone followed by a second 100 nM OT stimulus. In Fig. 5B, a similar experiment is shown using 1 μM RU 28362. Five identified corticotropes responded in the same way in the presence of the glucocorticoid agonist. Note that a similar protocol was followed in secretion studies where the cells were exposed to steroids for 25 min before and 5 min during OT stimulation. In all cells analyzed, the OT-induced intracellular calcium transients were not substantially different when the steroids were present.

Discussion

The regulation of adenohypophyseal ACTH secretion is complex and involves multiple regulatory factors of hypothalamic, neurohypophyseal, and peripheral origins. Among the stimuli that elicit ACTH from pituitary corticotropes are CRF and AVP (for review see Ref. 1). Recently, OT was shown to be an effective secretagogue of ACTH secretion: when applied in pulsatile fashion, it induces ACTH release by isolated pituitary corticotropes dose dependently and independent of the presence of CRF or AVP (6). An increase of intracellular calcium by mobilizing calcium mainly from intracellular stores appears to be the basic mechanism of OT action leading to ACTH secretion.

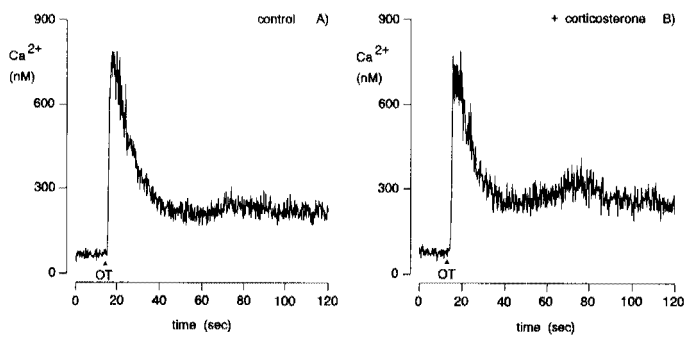

FIG. 4. Rise of OT-induced intracellular calcium in a single immunocytochemically identified corticotrope in the absence or presence of corticosterone. Intracellular calcium concentration rapidly increases by a challenge with OT (100 nM) (A). After washing and preincubating the cells with 1 μM corticosterone for 25 min, the OT-responding cells were challenged with a second OT stimulus (B). *Arrowheads* indicate additions of OT. The profiles shown are representative of six cells treated in the same manner.

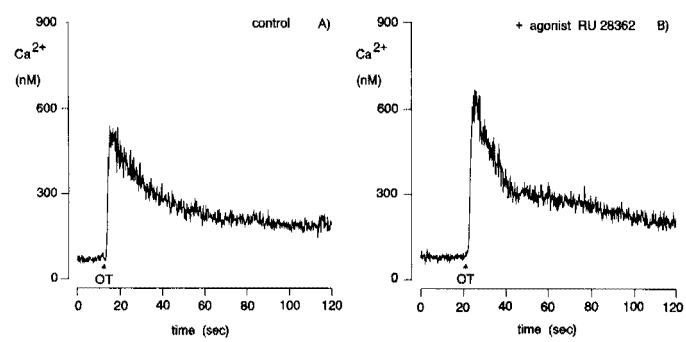

FIG. 5. Intracellular calcium levels in response to OT (100 nM) in the absence or presence of RU 28362. A similar experimental protocol was followed as described in Fig. 4. The glucocorticoid agonist RU 28362 (1 μM) was present 25 min before the second OT stimulus. *Arrowheads* indicate additions of OT. The profiles shown are representative of five cells treated in the same manner.

As is generally accepted, increases in cytosolic free calcium result in the release of hormones or neurotransmitters by exocytosis (12–15, 29, 30). Such increase in intracellular calcium are brought about by calcium influx through the plasma membrane and/or release of calcium from intracellular stores (for review see Ref. 31). AVP causes the breakdown of phosphoinositides and formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol in dispersed rat anterior pituitary cells (32). IP_3 causes an increase in cytosolic calcium by evoking its release from intracellular stores from many different types of cells including endocrine cells (33, 34). Experimental evidence reported by different groups, that is secretion studies in the presence or absence of extracellular calcium or direct measurements of calcium transients in corticotropes exposed to OT or AVP (6, 9, 35, 36), emphasizes the involvement of intracellular calcium stores during ACTH release. On the other hand, CRF causes influx of extracellular calcium through calcium channels, and these influxes are secondary to CRF-stimulated cAMP production (8, 37, 38). It has been proposed that calcium channels in a variety of cells are activated by cAMP (39, 40). A study with permeabilized corticotrope tumor cells (AtT-20) provides evidence for requirement of calcium and no direct effect of cAMP on exocytotic release of ACTH (16). Thus, it can be concluded that intracellular calcium is the main regulatory substance, which triggers ACTH release from corticotropes irrespective of the secretagogues. However, as outlined above, the calcium sources are different: signal pathways for AVP and OT lead to mobilization of calcium mainly from intracellular stores, whereas CRF stimulation leads to calcium influx.

It is well documented that inhibition of ACTH secretion is exerted by glucocorticosteroids from the adrenal cortex, which constitutes the negative feedback loop of the hypothalamo-pituitary-adrenocortical axis. Three types of time domains of the inhibitory action—rapid, delayed, and slow suppression—can be distinguished, and the rapid inhibitory action was proposed to be an effect on the secretory process (17). Although POMC gene down-regulation by glucocorticoids has been observed within 15 min, an acute reversible change in the amounts of translated ACTH available for release in corticotropes is not likely (41, 42). The rapid inhibition by glucocorticoids of CRF- or AVP-induced ACTH release is well documented (18, 21, 43, 44). The present investigation demonstrates the rapid inhibition by glucocorticoids of OT-stimulated ACTH secretion. The time course (suppression is evident within 30 min) as well as the degree of inhibition (60%) is consistent with previous findings with other secretagogues (19, 45). The independence of rapid inhibition of the secretagogue used to stimulate ACTH release and therefore of the intracellular pathways, which lead to elevation of cytosolic calcium, suggests a common mechanism of the glucocorticoid effect. In addition, our results

imply that rapid inhibition of OT-induced ACTH release by glucocorticoids, similar to the release induced by CRF or AVP in earlier studies (21), is mediated through glucocorticoid receptors.

In contrast to a previous study (35), where 2 h dexamethasone-pretreated adenohypophyseal cells were exposed to a single stimulus (10 min), we observed during pulsatile applications of secretagogues a pronounced effect of glucocorticoids on the peak of ACTH released. In addition, the short exposure (30 min) to corticosterone or glucocorticoid agonist and antagonist indicate the potency and specificity of the steroid effects on ACTH secretion due to pulsatile application of secretagogues. Such an experimental procedure is closer to conditions *in vivo*, as evident from previous work demonstrating the rhythmic activity of oxytocinergic neurons in the paraventricular nucleus (46) and pulsatile release of OT into the circulation (47).

However, the mechanism of rapid glucocorticoid inhibition on stimulated ACTH secretion is still unclear. In our previous study (6), we showed direct evidence that OT-induced ACTH secretion implies mobilization of calcium from intracellular stores. Our present results clearly demonstrate that the inhibitory effect of glucocorticoid on OT-induced ACTH secretion is not exerted through changes in intracellular calcium levels, since neither glucocorticoid (corticosterone or RU 28362) exhibits an effect on the OT-induced calcium transients. In a previous study it was shown that AVP-induced calcium rise was also not altered by the presence of dexamethasone from normal pituitary cell suspension (48). Since neither the kinetics of the OT-induced calcium transient onset nor the height of the peak were affected by the presence of glucocorticoids in the present study, we conclude that cytosolic calcium transients induced by OT are not affected by glucocorticoids, suggesting that glucocorticoids may not inhibit IP_3-mediated calcium release from intracellular stores. Furthermore, IP_3 production induced by physiological concentrations of AVP in anterior pituitary fragments is not inhibited by glucocorticoids within the rapid time domain (49, 50). These data suggest an effect of glucocorticoids on a step further downstream in the stimulus-secretion coupling process, *i.e.* beyond stimulus-induced calcium mobilization from intracellular stores.

The reports on glucocorticoid effects on cAMP production are controversial: An alteration of CRF-induced increases of cAMP levels by glucocorticoids was proposed (49, 51) and refuted (23, 52, 53). The latter studies, which support an effect of glucocorticoids beyond the rise of cAMP, are in accordance with secretion studies using permeable analogs of cAMP (20, 54).

Studies using the clonal pituitary cell line AtT-20 also provide contradictory results regarding the glucocorticoid effect on CRF-induced intracellular calcium increase. A suppression of CRF-induced calcium transients was shown (22), whereas in another study no effect of glucocorticoids on intracellular calcium rise was found (23). A direct effect of glucocorticoids on stabilization of actin filaments was suggested as a possible mechanism (23). However, the slow time course for any rearrangement of the cytoskeleton (2 h) does not correspond to the rapid glucocorticoid inhibition of ACTH release by OT, CRF, and AVP reported in the present and previous studies (21, 44).

Taken together, we provide direct evidence that OT-induced ACTH secretion is inhibited by glucocorticoids rapidly and that the immediate inhibitory action does not alter OT-induced calcium transients in corticotropes. We conclude that the inhibitory action on exocytotic ACTH release is mediated through glucocorticoid receptors and is exerted beyond the step of intracellular calcium increase.

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