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Glucocorticoid stabilization of actin filaments: A possible mechanism for inhibition of corticotropin release

(exocytosis/pituitary/secretory granules/cytochalasin)

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ABSTRACT **The mechanism by which glucocorticoids induce various cellular responses in different tissues is only partially understood. Here we demonstrate that glucocorticoids stabilize the actin cytoskeleton of several cell types, as revealed by increased resistance of actin filaments to the disrupting effect of cytochalasin and by visible thickening of actin filament bundles. These effects require several hours to develop, require protein synthesis, and are accompanied by increased expression of the actin-binding protein caldesmon. These data may help to explain why glucocorticoids inhibit corticotropin release from pituitary cells, if interpreted in terms of the current idea that an actin filament "barrier" modulates exocytotic secretion in various cell types. In support of this idea, we find that in "model" corticotrophs (AtT-20 cells), glucocorticoids stabilize actin filaments and inhibit corticotropin release with similar potencies. Furthermore, we show here that glucocorticoid inhibition is overcome by exposing AtT-20 cells to concentrations of cytochalasin B or D that disrupt their stabilized actin filaments. On the other hand, our freeze-etch electron microscopy of AtT-20 cells has shown that actin filaments do not, in fact, create a dense submembranous barrier that might prevent corticotropin secretory droplets from discharging; instead, they form open networks near the membrane that appear to hold secretory droplets in their interstices. We propose that the delicate physical crosslinks maintaining this actin-mediated membrane "docking" of secretory droplets may need to disconnect in order to permit corticotropin discharge and that these crosslinks may be stabilized along with the actin filaments in dexamethasone-treated cells.**

Glucocorticoids act by transcriptionally activating certain sets of genes in susceptible cells, but the subsequent steps that link this altered gene expression to the various cellular changes induced by glucocorticoids are generally unknown (1). In this report, we analyze the inhibitory effect of glucocorticoids on corticotropin (ACTH) release from pituitary cells. Studies of primary pituitary cultures and of pituitary tumor cells have shown that glucocorticoid-induced inhibition of ACTH secretion has two phases, one appearing in a few hours and the other requiring days of exposure to steroids. The latter, long-term effect is known to involve a decrease in ACTH synthesis, but the former, rapid-onset suppression of exocytotic release of hormone is less well understood (2, 3). Primary pituitary cell cultures are unsuitable for studying this effect because corticotrophs comprise only 2–5% of the total cell population, so we used instead a clonal cell line (AtT-20) derived from a mouse tumor, which is well characterized and maintains several differentiated characteristics (4). As in normal corticotrophs, corticotropin-releasing factor (CRF) stimulates ACTH release from AtT-20 cells (4), and it does so by raising cAMP levels, leading to protein kinase A-mediated opening of voltage-dependent calcium channels, with a resulting increase in cytosolic calcium and triggering of exocytosis (5, 6). Glucocorticoids suppress CRF-induced release without inhibiting the increase in cAMP (4, 7) and also inhibit the ACTH release induced by postreceptor stimuli such as 8-Br-cAMP, forskolin, and phorbol 12-myristate 13-acetate (2, 4, 7). Thus, the inhibitory effects of steroids appear to be late in the stimulus–secretion cascade.

Several investigators have proposed that, in other secretory cells, a network of filamentous actin underlies the plasma membrane and regulates exocytosis by acting as a barrier that impedes the apposition of secretory granules to membrane fusion sites (for review, see refs. 8 and 9). For example, in chromaffin cells, the secretagogues nicotine and potassium appear to induce a fragmentation or reorganization of the cortical actin web in parallel with their stimulation of catecholamine release (8, 10–14), possibly by activating actin-severing proteins (15, 16). Similar conclusions have been offered for mast cells (17, 18). Furthermore, agents that stabilize or destabilize filamentous actin (F-actin) generally potentiate or inhibit secretion, respectively (17, 19, 20). Unfortunately, although the "barrier" theory accounts for the majority of the relevant data, it is clouded by imperfect understanding of the actual organization of the actin cytoskeleton in secretory cells.

Here we demonstrate that glucocorticoids stabilize filamentous actin in AtT-20 cells and that the inhibition of ACTH release can be correlated with the stabilization of actin filaments. We also show that glucocorticoids stabilize F-actin and increase expression of the actin-binding protein caldesmon in several other cell types. On the other hand, upon examination of dexamethasone-inhibited cells by freeze-etch electron microscopy, we fail to find a confluent actin barrier to secretion of the kind previously hypothesized (21). Instead, we find that secretory droplets, in this cell type at least, are in contact with the plasma membrane and in fact are held in that position by lateral connections to surrounding but nonintervening actin filaments. Hence, we propose a modification of the barrier theory, by which glucocorticoid stabilization of these "encompassing" actin filaments or of the delicate connections they make with secretory droplets may somehow retard normal ACTH discharge.

MATERIALS AND METHODS

Cell Culture. AtT-20/D16-16 cells were grown as described (22). Human pneumocytes type II (A549) were cultured in modified Eagle's minimal essential medium with 10% fetal calf serum, in 5% CO_2/95% air.

ACTH Secretion. The experiments were carried out as described (22).

Biochemical Assays. ACTH and cAMP were measured by radioimmunoassay as described (22). Caldesmon was as-

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Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; F-actin, filamentous actin.

sayed by quantitative immunoblot and densitometry (LKB Ultroscan XL) (23).

Fluorescence Microscopy. Cells were plated in 24-well Falcon dishes coated with gelatin or poly(D-lysine) (0.1 mg/ml) for 1 hr at a density of 1×10^4 cells per dish, fixed in 2% paraformaldehyde, permeabilized with 0.02% Triton X-100 in phosphate-buffered saline, washed, and stained with rhodamine/phalloidin.

Freeze-Etch Electron Microscopy. Cells were plated and prepared for transmission electron microscopy as described by Heuser (24).

Cytosolic Calcium in Single Cells. Cells attached to glass coverslips were loaded with 1 μM fura-2 for 30 min at 25°C, washed, and transferred to a perfusion chamber mounted on the stage of a Zeiss IM microscope where cells were continuously superfused in a stream of Hanks' salt solution. Cytosolic [Ca^{2+}] was measured by a Spex DM3000 CM system according to published procedures (25).

RESULTS

Stabilization of Actin Filament by Glucocorticoids. *Effect of cytochalasins.* As shown in Fig. 1*a* by light microscopy, most filamentous actin in AtT-20 cells appears to be subjacent to the plasma membrane. Cytochalasins D and B (fungal toxins that cap the barbed end of actin filaments; see ref. 26) disrupt this subplasmalemmal actin and induce the appearance of lumps of phalloidin-binding material in the cell interior. The effect of 5 μg of cytochalasin D per ml for 10 min is shown in Fig. 1*c*. The time course of the toxin's effect is shown in Fig. 2. Lower concentrations of cytochalasin D (0.5 μg/ml) produce similar results with a slower time course (see legend to Fig. 2). Cytochalasin B is approximately as potent as the D analogue (see legend to Fig. 2). Trypan blue tests indicate that cell viability is not affected by the cytochalasins. Moreover, upon removal of cytochalasins, the disruption of the actin cytoskeleton is completely reversed within 60 min (data not shown).

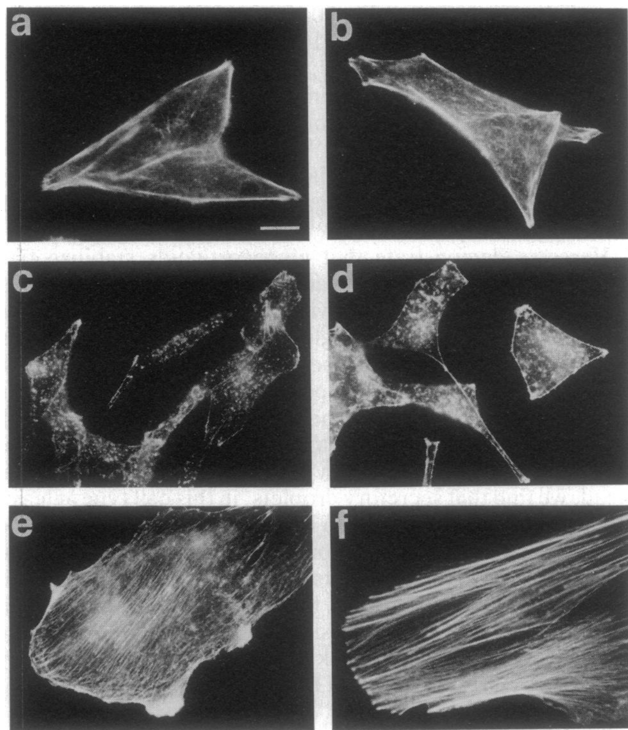

FIG. 1. Dexamethasone protects actin filaments from cytochalasin-induced disruption and increases thickness of actin bundles. AtT-20 (*a–d*) and A549 (*e* and *f*) cells were exposed to dexamethasone (0.1 μM in *b* and *d*, 1 μM in *f*) during the last period of growth (4–15 hr for AtT-20 and 48 hr for A549 cells), washed, exposed to cytochalasin D (5 μg/ml) for 10 min in Dulbecco's modified Eagle's medium/bovine serum albumin (*c* and *d*), washed again, fixed, and stained with rhodamine/phalloidin. Cytochalasin B (5 μg/ml for 10 min) caused similar effects (four experiments). Dexamethasone and cytochalasins were diluted 1:1000 from stock solutions in ethanol and dimethyl sulfoxide, respectively; vehicles had no effect on actin filaments. (*a* and *b*, bar = 3 μm; *c* and *d*, bar = 3.5 μm; *e* and *f*, bar = 4 μm.)

Effects of glucocorticoids on actin filaments. Pretreatment with dexamethasone (0.1 μM for 15 hr) partially protected AtT-20 cells against cytochalasin-induced disruption of actin filaments (Figs. 1*d* and 2); however, this protection could be overcome by prolonged exposure to cytochalasins (Fig. 2). This dexamethasone effect required time to develop; it became detectable after 2 hr of treatment (in two of three experiments; data not shown) and was maximal after 12–15 hr (see results in Figs. 1*d* and 2).

In terms of potency, the effects of dexamethasone were maximal at 0.1 μM (if applied for 15 hr) and were barely detectable at 1 nM. Less potent were betamethasone (threshold, 10 nM; maximal effect, 1 μM) and hydrocortisone (threshold, 0.1 μM), while progesterone, β-estradiol, and testosterone were all inactive at up to 1 μM.

A second notable effect of dexamethasone was to induce thickening of actin bundles. In AtT-20 cells, this response was variable and generally small but detectable (Fig. 1*b* exemplifies a slight thickening effect), while in the A549 cell line it was marked and reproducible (Fig. 1*f*). The thickening effect in A549 cells became apparent after 12 hr of treatment with 1 μM dexamethasone and was maximal after 24 hr (exemplified in Fig. 1*f*). NRK cells also showed glucocorticoid-induced thickening of actin bundles after a similar treatment, while PC12 cells were unaffected (data not shown). Of the two effects of dexamethasone in AtT-20 cells, the protection against cytochalasin-induced disruption of actin filaments appeared earlier than filament thickening

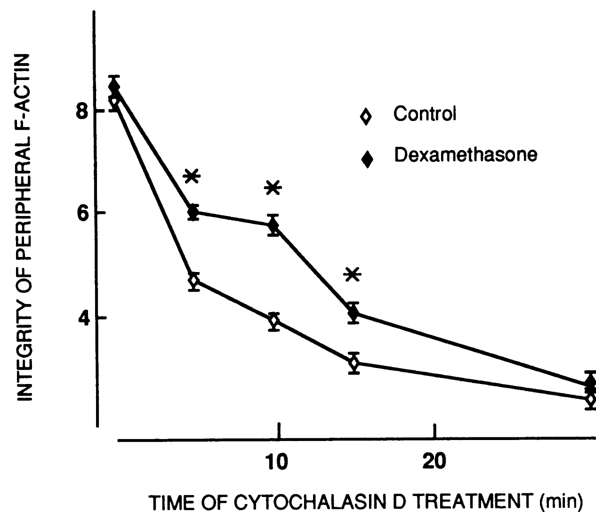

FIG. 2. Time course of effects of cytochalasin on peripheral actin filaments in control and dexamethasone-treated AtT-20 cells. Dexamethasone (0.1 μM) and cytochalasin D (5 μg/ml, for the specified times) were used as described in the legend to Fig. 1. The effect of cytochalasin D was quantified by a trained technician in a blind fashion according to the following procedure: 30 randomly chosen cells from every experimental treatment (15 cells from each duplicate) were examined at ×1000 magnification under a Zeiss Axiophot fluorescence microscope and were given individual scores from 0 to 10, using as a criterion the integrity of the peripheral actin meshwork. For instance, a cell with ≈80% of its margins fluorescently stained with phalloidin received a score of 8. The presence of fluorescent spots in the cell interior did not influence the score. A treatment with 0.5 μg of cytochalasin D per ml for 30 min produced effects that were similar to those induced with 5 μg/ml for 10 min. Values represent means ± SE of 30 individual scores. *, Different from controls by Duncan's test ($P < 0.01$). Similar results were obtained in three experiments.

(after 2–3 vs. 12 hr of treatment) and was evident even in experiments in which the latter effect was not noticeable.

We next examined whether the stabilization of actin filaments might be explained by changes in the cellular levels of actin-stabilizing proteins, such as tropomyosin and caldesmon (27, 28). Indeed, in A549 cells, dexamethasone (1 μM for 24 hr) induced a marked increase (550% ± 50%, average of six experiments) in the concentration of caldesmon, as measured by immunoblot analysis. The increase in AtT-20 cells was less marked (160%). The concentrations of total cellular actin, gelsolin, and tropomyosins were not modified by glucocorticoids, indicating a degree of specificity for the caldesmon increase.

As a further control of the specificity of dexamethasone action, we tested whether the steroid had effects on microtubules; neither the apparent amount nor the distribution of microtubules (immunofluorescence data) was affected by the steroid (at 0.1 μM for 15 hr under the conditions described in the legend to Fig. 2; data not shown) in AtT-20 cells and in A549 cells.

Role of Actin Filament Stabilization in Inhibition of ACTH Secretion. The concentrations of glucocorticoids that inhibit ACTH release in AtT-20 cells were similar to those found to stabilize actin filaments (EC_{50} values: dexamethasone, ≈3 nM; betamethasone, ≈10 nM; hydrocortisone, ≈0.1 μM), suggesting that the two effects might be related. To substantiate this possibility, we sought to overcome dexamethasone stabilization of actin filaments to then determine whether the steroid still inhibited CRF-stimulated ACTH release. This was accomplished by prolonging the exposure of dexamethasone-treated cells to cytochalasin D to 30 min, at which point actin filament disruption became as complete as in control cells (see Fig. 2). Indeed, the block of ACTH secretion was largely overcome (Fig. 3). Cytochalasins did not potentiate CRF stimulation in control cells nor did they modify basal ACTH release in control or in dexamethasone-treated cells.

Specificity of the cytochalasin D effects. We tested whether the cytochalasin potentiation of CRF-induced ACTH release in dexamethasone-treated cells might be due to effects on the second messengers induced by CRF. Cytochalasins did not increase the CRF-stimulated synthesis of cAMP in dexamethasone-treated cells (Table 1); rather, they tended to decrease it. Likewise, they did not modify cytosolic [Ca^{2+}] (Table 1). To investigate further possible nonspecific effects of cytochalasins, we also carried out control experiments with somatostatin, a powerful inhibitor of ACTH secretion in AtT-20 cells, but one whose mechanism of action appears to be quite different from that of glucocorticoids (4, 29, 30). Somatostatin did not increase the stability of actin filaments in AtT-20 cells (fluorescent phalloidin; data not shown), nor did the cytochalasins ameliorate inhibition by somatostatin of ACTH release to a degree comparable to that found for dexamethasone (Fig. 3). However, a partial relief of inhibition by somatostatin was found in some experiments, which might be due to slight nonspecific effects of the toxin. Evident and reproducible effects of cytochalasin on CRF-stimulated ACTH release were restricted to cells in which the actin cytoskeleton had been overstabilized by dexamethasone.

Spatial relationships between granules and actin filaments. To further pursue the notion that stabilization of actin filaments might be involved in the inhibition of ACTH release, we examined the spatial relationship between actin filaments, the plasma membrane, and the secretory granules. These experiments were performed by applying the freeze-etch technique to cells that were broken open by a quick burst of sonication (24). Figs. 4 and 5 show examples of the images obtained. They display the bottoms of cells that were broken open and decorated with elements that remain attached to the inside of the plasma membrane. Secretory granules, when they occur, are generally found in clusters. Typically, these clusters are found at the distal ends of cell processes, in agreement with previous views (31, 32). Within the clusters, granules are linked to each other and to surrounding actin

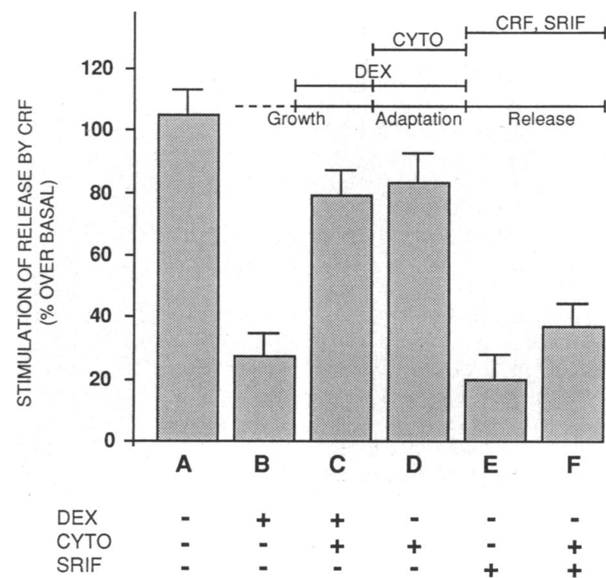

FIG. 3. Cytochalasin reverses dexamethasone-induced inhibition of CRF-stimulated ACTH release in AtT-20 cells. Cells were exposed to 0.1 μM dexamethasone (DEX) during the last 4 hr of growth as well as during the adaptation period. Cytochalasin D (CYTO) (5 μg/ml) was present, when appropriate, during the adaptation period, and CRF and somatostatin (SRIF) (both at 0.1 μM) were applied (simultaneously, when appropriate) at the beginning of the release period (30 min). The medium was then removed and assayed for ACTH. All agents were diluted from 1000-fold concentrated stock solutions in dimethyl sulfoxide (DEX and CYTO) or bovine serum albumin/saline (SRIF). Vehicles had no effect on ACTH secretion. The effect of CRF on ACTH release is expressed as percentage stimulation over the appropriate basal value according to the formula {[(CRF-induced release)/(basal release)] × 100} − 100. Basal release values were as follows: A, 13 ± 1.2; B, 12.1 ± 1.1; C, 13.8 ± 2.7; D, 11.2 ± 2; E, 11 ± 1.6; F, 12.1 ± 1.9 (ng per well). Bars represent means ± SE of 18 determinations carried out in six experiments.

Table 1. Effects of cytochalasin D on cytosolic [Ca^{2+}] and [cAMP] in CRF- and dexamethasone-treated AtT-20 cells

Treatment	[Ca^{2+}], nM	[cAMP], pmol per well
Control	107 ± 9	7.8 ± 0.7
CRF	156 ± 11	47.1 ± 4
DEX	102 ± 8	7.1 ± 0.3
DEX–CRF	144 ± 18	39.5 ± 3
CYTO	110 ± 12	8.4 ± 0.6
CYTO–CRF	155 ± 10	33.6 ± 2
CYTO–DEX	104 ± 11	6.1 ± 0.2
CYTO–DEX–CRF	150 ± 16	30.3 ± 2

Dexamethasone (DEX), cytochalasin (CYTO), and CRF were used exactly as described in the legend to Fig. 3. For cAMP experiments, 0.5 mM isobutylmethylxanthine was added during the adaptation and release periods to inhibit cAMP degradation. cAMP values are means ± SE of nine determinations from three experiments. Cytosolic [Ca^{2+}] was measured in single cells by the fura-2 technique. Basal [Ca^{2+}] levels were stable; CRF induced a [Ca^{2+}] increase, which peaked 90 ± 11 s after application of the hormone. Peak [Ca^{2+}] levels were used to quantify the CRF stimulatory effects. [Ca^{2+}] values are means ± SE of at least 12 different measurements obtained on three different occasions. In 4 of 17 cells of the DEX–CRF group, the [Ca^{2+}] increase was inhibited by >50%, while in the remaining 13 it was within the control range, perhaps suggesting the existence of a small DEX-sensitive cell population. All agents were diluted from 1000-fold concentrated stock solutions. Vehicles had no effects.

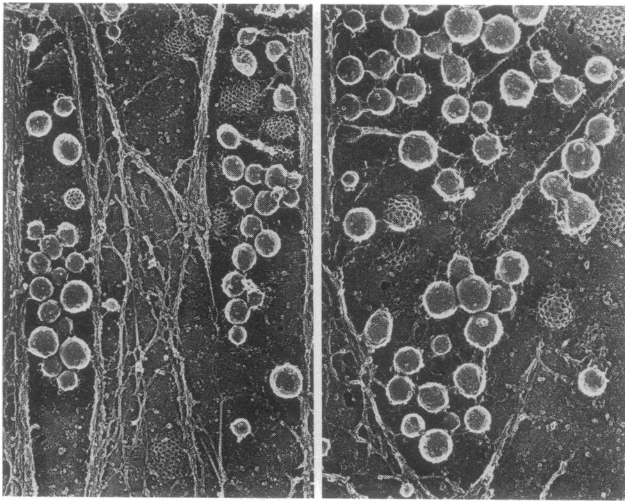

FIG. 4. Transmission electron microscopic views of platinum replicas of freeze-dried AtT-20 cell interiors after ultrasonic disruption and removal of overlying cytoplasm. Spherical secretory droplets ≈0.1 μm in diameter remain attached to the inner surface of the plasma membrane, apparently by thin fibrils that crosslink them to the membrane as well as by other crosslinks that attach them to surrounding bundles of actin filaments. Actin filaments do not intervene between secretory droplets and the plasma membrane.

filaments by short fibrils (Figs. 4 and 5). Contrary to the actin barrier hypothesis, granules do not appear to be held away from the plasma membrane by their attachments to actin, nor do the actin filaments directly intervene. In other words, the subplasmalemmal actin filaments are never sufficiently dense to create a confluent meshwork that could form a physical barrier between granules and the membrane, neither in dexamethasone-treated nor in control cells. Instead, granule clusters occur mostly in the open areas between actin filament networks, in contact with the plasma membrane or separated from it by only a thin intervening layer of fine fibrils no thicker than those that attach granules to each other or to surrounding actin filaments. Glucocorticoids did not have apparent effects on these structures.

DISCUSSION

The main findings reported here are that (*i*) glucocorticoids stabilize actin filaments in diverse cell types including AtT-20 corticotrophs, and (*ii*) stabilization of actin filaments correlates with, and is involved in, inhibition of ACTH secretion in AtT-20 cells.

Actin Filament Stabilization by Glucocorticoids. Actin filaments are dynamic structures whose overall aboundance is related to the balance of polymerization and depolymerization

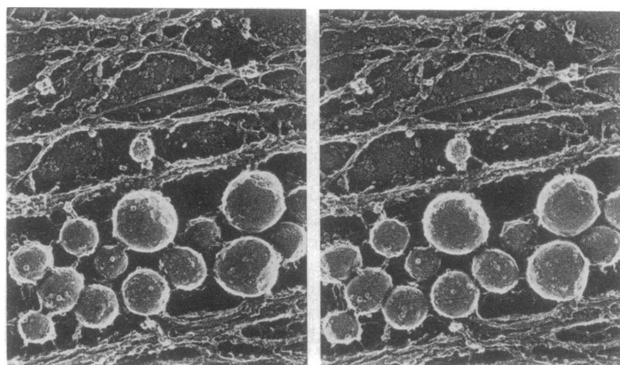

FIG. 5. Stereoview of relevant areas in Fig. 4 to clarify the critical disposition of the fine crosslinking fibrils vis-à-vis the ACTH secretory droplets. See text for discussion.

at the filament ends as well as to the number of ends available for such exchange. Filaments that frequently expose new ends and turn over rapidly are more likely to be disrupted by cytochalasin than are stable ones. For instance, actin filaments involved in a dynamic cellular activity such as movement are more sensitive to cytochalasins than are those supporting cell shape or tensile strength (33). Thus, we propose that the glucocorticoid effect on actin filaments is to increase their stability (Fig. 1), probably by reducing F-actin breakdown and/or by reducing the formation of filament ends. Reduced actin breakdown would also be in line with the observation of thicker actin bundles in dexamethasone-treated cells (see Fig. 1*f*), although bundle thickening could also be explained by a reorganization of preexisting filaments. Dexamethasone protection is reproducible and highly statistically significant but partial (the maximal effect being a 38% reduction of the cytochalasin effect). We do not propose, however, to correlate quantitatively the anticytochalasin action of dexamethasone with its release-suppressing effect. It is quite possible, and to our mind likely, that the physiological mechanisms that may control the disassembly of actin filaments *in vivo* during secretion might act in a more delicate manner than a toxin such as cytochalasin and that the protection afforded by glucocorticoids against such mechanisms might be more complete than the protection against cytochalasin. One possible mechanism underlying these effects of glucocorticoids could be related to the observed increase in caldesmon levels. Caldesmon is an actin- and calmodulin-binding protein that regulates interactions between actin, tropomyosin, and myosin (27). In particular, caldesmon greatly potentiates the ability of tropomyosin to inhibit the actin-severing action of gelsolin and to reanneal already severed F-actin (28). Experiments involving manipulations of expression of the caldesmon gene are required to test this hypothesis.

Role of Actin Filament Stabilization in Inhibition of ACTH Release. The finding that several different glucocorticoids (but not other steroids) commensurately stabilize the actin cytoskeleton and inhibit ACTH release in AtT-20 cells, and do so with similar potencies, suggests that the two effects are related. The main evidence in favor of this proposal is that cytochalasin, at filament-disrupting doses, relieves the dexamethasone inhibition of ACTH release. This effect does not appear to be mediated by mechanisms other than microfilament disruption, since cytochalasin does not increase the levels of the second messengers mediating CRF-induced ACTH release (5, 6) nor does it nonspecifically potentiate secretion in control cells. Furthermore, the inhibitory effect of somatostatin, which does not appear to involve F-actin stabilization, is just barely modified by cytochalasin.

The above results might seem consistent with the hypothesis that in AtT-20 cells a cortical actin meshwork could form a barrier that limited the approach of secretory granules to the plasma membrane (21) and that such a barrier would be broken down by CRF except when stabilized by glucocorticoids. However, the freeze-etch images of AtT-20 cells presented here do not support such a barrier model in its simple form. They show that in cell processes in which secretory granules collect, actin filaments do not intervene between them and the plasma membrane but instead appear to hold them in their interstices in contact with the membrane. This fits with previous thin-section images obtained by conventional transmission electron microscopy, showing secretory granules in AtT-20 cells adjacent to the plasma membrane (31, 34). It has been proposed that short, Triton-soluble actin filaments are involved in mast-cell secretion (17); our results make it unlikely that such structures might play a role in AtT-20 cells. Instead, they indicate that in these cells actin filaments might modulate exocytosis in a more complex manner than previously proposed. We suggest that actin filaments control granule fusion not by physically

intervening between granules and plasma membrane, as proposed by the barrier hypothesis, but by holding the granules away from, although close to, their final discharge sites. The fact that granules are trapped in a position adjacent to the plasma membrane, presumably near their fusion sites, might represent a predocking preparatory step that could facilitate secretion when CRF would induce a controlled dissolution of the actin network. Filament disassembly would thus be necessary (but not sufficient, as shown by the lack of effect of cytochalasin on basal release) for secretion. The same structure, however, might impede secretion if overstabilized by glucocorticoids. This scheme would account for the observations that disassembly of the actin network by cytochalasin in dexamethasone-treated cells relieves the secretory paralysis, while in control cells it results in a slight inhibition, rather than facilitation, of CRF-induced release (see Fig. 3). A similar model has been proposed by Landis *et al.* (35) and by Hirokawa *et al.* (36) regarding the interrelationships among secretory vesicles, synapsin, actin, and spectrin in presynaptic terminals. Glucocorticoids would stabilize the actin meshwork by inducing the synthesis of a protein(s) such as caldesmon. It should be noted that caldesmon might have other roles in secretion, since it can bind to both granules and actin filaments and has been proposed to be a linker protein between these two structures (37). It is also worth noting that the lack of glucocorticoid effects on the relationships between secretory vesicles and actin filaments rules out a mechanism of action of these steroids involving an altered disposition of granules, which could not be formally excluded before.

It is quite possible that a complete actin barrier might exist in chromaffin or mast cells. These cells are very different from pituitary cells: the former "explosively" secrete large fractions of their content within a few minutes, whereas the latter release at slow rates for long periods of time. It would not be surprising to find a different physical arrangement and/or regulation of filamentous actin in these cell types. For instance, the massive actin destabilizing effect of secretagogues observed in chromaffin cells is unlikely to be found in AtT-20 cells, where one might rather expect small local effects that would be difficult to detect.

Glucocorticoids inhibit exocytosis from several other cell types, including pituitary luteotrophs, neutrophils, and mast cells (38–40). As actin filament stabilization is the most economical hypothesis to explain the glucocorticoid effects in AtT-20 cells, we propose that it could apply to these other cells as well. Furthermore, the present data raise the interesting possibility that reduction in actin filament plasticity might be involved not only in inhibition of secretion, but also in other glucocorticoid effects, such as inhibition of fibroblast and macrophage motility (41) and inhibition of the cytoskeletal reorganization that precedes cytolysin secretion in natural killer lymphocytes (42).

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