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Depolarization counteracts glucocorticoid inhibition of adenohypophysical corticotroph cells*

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1 In AtT20 mouse corticotroph tumour cells large conductance Ca^{2+}-activated K^+-channels (BK-channels) have an essential role in the early glucocorticoid inhibition of adrenocorticotrophin (ACTH) secretion evoked by corticotrophin-releasing factor. The present study examined whether or not BK-channels are also pivotal to glucocorticoid inhibition of normal rat anterior pituitary cells.

2 A membrane-permeant, non-metabolizable cyclic AMP analogue, 8-(4-Chlorophenylthio)adenosine-3′,5′-cyclic-monophosphate (CPT-cAMP) was used as the primary secretagogue stimulus, as this mimics the increase of intracellular cyclic AMP caused by corticotrophin-releasing factor, but is not subject to the complex Ca^{2+}-dependent regulation of cyclic AMP metabolism that is evident in corticotroph cells.

3 Experiments in AtT20 cells showed that ACTH secretion stimulated by 1 mM CPT-cAMP was suppressed to $34 \pm 1.5\%$ ($n=12$) of the control stimulus by a maximal dose of 100 nM dexamethasone. The ACTH secretion evoked by the combination of 1 mM CPT-cAMP with either 5 μM (−)BayK8644 (L-type Ca^{2+}-channel activator) or 5 mM TEA (K^+-channel blocker) was respectively $69.1 \pm 7.6\%$ and $69.3 \pm 11.8\%$ of control after 2 h preincubation with 100 nM dexamethasone ($P<0.05$ *vs* CPT-cAMP). The ACTH response elicited by 5 μM (−)BayK8644 and 5 mM TEA given together was completely resistant to inhibition by 100 nM dexamethasone. Furthermore, TEA and (−)BayK8644 given together synergistically stimulated ACTH release in combination with 0.1 mM or 1 mM CPT-cAMP, and these ACTH responses were not inhibited by 100 nM dexamethasone.

4 In primary cultures of rat anterior pituitary cells, TEA (up to 20 mM), charybdotoxin (30 nM) or apamin (100 nM) failed to modify the glucocorticoid inhibition of 0.1 mM CPT-cAMP-induced ACTH release. The combination of 5 mM TEA and 5 μM (−)BayK8644 elicited a small but significant increase in ACTH secretion but did not modify the inhibition of 0.3 mM CPT-cAMP-induced ACTH secretion by 100 nM dexamethasone.

5 In primary cultures of rat anterior pituitary cells, depolarization of the membrane potential with 40 mM KCl enhanced the ACTH response to CPT-cAMP and markedly reduced the maximal inhibitory effect of dexamethasone to $55 \pm 1.2\%$ as well as that of corticosterone to $33 \pm 2.1\%$ *vs* $100 \pm 2.5\%$ and $100 \pm 1.9\%$ inhibition respectively, when 0.1 mM CPT-cAMP was used alone. Introduction of 5 μM (−)BayK8644 with 40 mM KCl in this system had no additional effect on glucocorticoid inhibition.

6 No glucocorticoid inhibition of ACTH release to any of the stimuli applied was observed in cells pretreated with the mRNA synthesis inhibitor, 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) (0.1 mM) or the protein synthesis blocker, puromycin (0.1 mM).

7 In summary, early glucocorticoid inhibition of stimulated ACTH release by cultured rat anterior pituitary cells was dependent on the synthesis of new mRNA and protein. Depolarization of the membrane potential potentiated CPT-cAMP-induced ACTH secretion in AtT20 cells as well as cultured rat corticotrophs and this was associated with a resistance to the early inhibitory effect of glucocorticoids. Glucocorticoid inhibition in rat anterior pituitary corticotrophs was unaltered by TEA, charybdotoxin as well as apamin, and hence it is unlikely to involve predominantly BK-or SK-type Ca^{2+}-activated K^+-channels.

8 These results support the thesis that a prime target of glucocorticoid feedback inhibition in anterior pituitary corticotrophs is the membrane potential and indicate that glucocorticoid-induced proteins regulate the activities of several distinct plasma membrane ion channels.

Keywords: Pituitary gland; potassium channels; dexamethasone; corticosterone; AtT20 cells; stress

Introduction

Adrenal corticosteroids are powerful regulators of gene expression in several organ systems of the body. The plasma levels of these steroids are controlled through a neuroendocrine feedback loop which ensures that corticosteroid levels are optimal for homeostatic adaptation (Dallman *et al*., 1992). Major sites of corticosteroid feedback action include the anterior pituitary gland, the hypothalamus and further sites in the central nervous system (Sapolsky *et al*., 1990).

The cellular mechanisms underlying corticosteroid feedback of the hypothalamic-pituitary-adrenocortical system are not well understood. The anterior pituitary corticotroph has served as a useful model to examine this problem (Antoni, 1996; Buckingham, 1996). In corticotroph cells corticosteroid inhibition typically occurs in two main phases, designated early (within 2 h) and late (2–24 h) (Antoni, 1996; Buckingham, 1996). Late inhibition appears to be mediated by negative regulatory elements in DNA and leads to the suppression of

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the proopiomelanocortin (ACTH precursor) (Lundblad & Roberts, 1988), the type 1 corticotrophin releasing-factor (CRF) receptor (Pozzoli *et al*., 1996) and possibly other genes. In contrast, the early component of inhibition is brought about by glucocorticoid-induced protein(s), the identity of which is unclear at present (Shipston, 1995; Antoni, 1996; Buckingham, 1996).

Recent studies in the mouse corticotroph tumour cell line AtT20 (Shipston *et al*., 1996) have shown that high conductance Ca^{2+}-activated K^+-channels (BK-channels) play an essential role in the inhibitory effect of dexamethasone on CRF-induced ACTH secretion. Briefly, activation of adenylyl cyclase by CRF produces an increase of cyclic AMP-dependent phosphorylation and triggers Ca^{2+}-dependent action potentials which lead to the exocytotic release of ACTH (see Antoni, 1993). An important element of this stimulatory effect is the inhibition of BK-channels by cyclic AMP-dependent protein kinase (Shipston *et al*., 1996). Treatment with dexamethasone for 90 min prevented the action of CRF and cyclic AMP analogues on BK-channels through a process requiring the synthesis of mRNA and protein (Shipston *et al*., 1996). Accordingly, the blockade of BK-channels by charybdotoxin (Antoni, 1996) or iberiotoxin (Shipston *et al*., 1996) obliterated the inhibitory effect of dexamethasone on stimulated ACTH release.

The aim of the present study was to examine whether BK-channels are also essential for corticosteroid feedback inhibition in non-tumoural corticotroph cells. Experiments were carried out *in vitro*, using AtT20 cells and primary cultures of rat anterior pituitary cells. A cell-permeant cyclic AMP analogue resistant to hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) was used as the stimulus in combination with various compounds that cause membrane depolarization. The rationale for this design was that increases of intracellular cyclic AMP induced by agonists are strongly suppressed by intracellular Ca^{2+} in both AtT20 and normal rat corticotrophs (Antoni *et al*., 1995; Antoni, 1997), hence changes of cyclic AMP metabolism induced by corticosteroids could mask effects downstream of cyclic AMP formation.

The results clearly show that in contrast to AtT20 cells, blockage of BK-channels fails to counteract early glucocorticoid inhibition in cultures of rat anterior pituitary cells. Importantly, however, corticosteroid inhibition in the rat pituitary culture system was markedly reduced upon depolarization with high extracellular KCl, in which respect its behaviour is analogous to AtT20 cells. Thus, the findings support the notion that early inhibition by glucocorticoids involves control of the membrane potential by rapidly induced proteins.

Methods

Reagents

Unless otherwise indicated all reagents were from Sigma U.K. (Poole, Dorset), and of the highest grade available. The sources of other materials were as follows: CRF (human), ACTH (human) synthetic charybdotoxin (Bachem U.K. Ltd., Saffron Walden); corticosterone (Roussell-UCLAF, Romainville, France); 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic-monophosphate (CPT-cAMP, BIOLOG, Bremen, Germany); tetraethyl ammonium hydrochloride (TEA, Aldrich, Gillingham, Dorset); (−)BayK8644, (RBI, Sigma, U.K.); (+)202-791 (Novartis, U.K.); TRL-3 trypsin (Worthington, Lorne Laboratories, Reading, U.K.); Sheep anti-ACTH serum was generously provided by Prof P.J. Lowry, University of Reading; anti-sheep/goat IgG donkey serum was generously provided by the Scottish Antibody Production Unit, Carluke, Scotland.

Cell culture

Male Wistar rats (120–200 g) were caged singly with free access to pelleted food and tap water in a light- and temperature-controlled environment for 24 h, and sacrificed by decapitation before 10.30 a.m. Precautions were taken to avoid any stressful stimulation of the animals. The anterior pituitary glands were collected and placed in a plastic Petri dish so that the ventral surfaces of the glands contacted the bottom of the dish. The glands were chopped twice (the second time after 90° rotation of the specimen platform) on a hand-operated McIlwain tissue-chopper setting 0.5 mm and lowest blade force. The tissue segments were incubated in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, GIBCO, Life Technologies, Paisley) supplemented with 25 mM HEPES pH 7.4, 0.15 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.5 mg ml^{-1} trypsin, 0.01 μg ml^{-1} DNAse I (Sigma D-4527) and 0.25% bovine serum albumin (BSA, Sigma A-7906) for 30 min at 37°C under constant shaking at 250 cycles/min, on a IKA Vibrax orbital shaker. Following trypsinization, 200 μl of 10^4 kallikrein unit/ml Trasylol® (Bayer U.K.) was added and the tissue segments were triturated with a 1 ml Gilson pipette tip attached to the end of a 5 ml polystyrene pipette for 10 min. The suspension was then filtered through 70 μM nylon mesh and centrifuged at 200 *g* for 10 min. The pelleted cells were resuspended in 5 ml HBSS supplemented with 25 mM HEPES pH 7.4, 0.15 mM $CaCl_2$, 0.15 mM $MgSO_4$, 0.01 μg ml^{-1} DNAse I, 0.25 mg ml^{-1} soybean trypsin inhibitor (Sigma T-6522), recentrifuged and suspended in Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g glucose/L) supplemented with 2.5% foetal calf serum (Harlan Seralab, Sussex) and 7.5% horse serum (Sigma H-1138). Cell viability as assessed by Trypan Blue exclusion was over 95%. Approximately 5×10^4 cells/well were plated in 24-well plates and cultured in a humidified incubator with 5% CO_2/95% air at 37°C for 4 days.

AtT20 cells were maintained in DMEM (containing 4.5 g/L glucose) supplemented with 10% foetal calf serum as previously described (Woods *et al*., 1992). All cells used were of passage 30 or less.

Assay of ACTH secretion

The experimental procedures for ACTH secretion were identical for AtT20 cells and primary cultures. The cells were first washed free of serum with 2×0.5 ml of incubation medium (DMEM, GIBCO 31600-026, without Na_2HCO_3, supplemented with 25 mM HEPES pH 7.4 and 0.25% BSA (Sigma, A-4378) and incubated in this medium at 37°C for 1 h, after which steroids and inhibitors of mRNA or protein synthesis were introduced, and incubation was continued for a further 2 h. Steroids were dissolved at 10^{-2} M in dimethylsulphoxide and diluted with incubation medium. Corresponding vehicle controls contained the requisite amounts of dimethylsulphoxide throughout.

In the case of primary cultures, K^+ channel blockers and Ca^{2+}-channel activators were added during the last 30 min of the 2 h preincubation period because normal corticotrophs appear to be relatively resistant to depolarization. After 2 h of preincubation, the wells were washed once more with

incubation medium and the secretagogues and requisite inhibitors were added in 0.5 ml of fresh incubation medium. In some experiments, high KCl medium which was composed of two parts incubation medium and one part 120 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$, 25 mM HEPES 0.25% BSA, Ca^{2+}-free medium (120 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4, 1.2 mM $MgSO_4$, 4.5 g/L D-glucose, 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, Minimum Essential Medium vitamins and amino acids) or Ca^{2+}-free high KCl medium (two parts of the Ca^{2+}-free medium mixed with one part of 120 mM KCl, 1.2 mM $MgSO_4$, 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, vitamins and amino acids) were used. The test incubation period was 60 min for primary cultures and 30 min for AtT20 cells after which the tissue culture trays were placed on ice. Any cells detached from the bottom of the wells were pelleted by centrifugation at 200 *g* for 10 min at 4°C after which aliquots of the supernatant were collected and assayed for ACTH content by radioimmunoassay. Sheep anti ACTH serum used in the assay was generously provided by Prof P.J. Lowry, University of Reading and the donkey antisheep IgG serum was obtained from the Scottish Antibody Production Unit, Carluke, Scotland.

Analysis of results

Each experiment was carried out at least twice with **n** replicates for each treatment within each experiment. All data are presented as mean ± s.e.mean and were analysed by 2- or 1-way analysis of variance followed by Dunnett's test, Newman Keuls' test or contrast of means where appropriate. Results presented as percentage of control ACTH release were derived from the raw data as follows: $(X-B)/(C-B)\times 100\%$, where B refers to the basal ACTH secretion, C refers to ACTH secretion elicited by the control stimulus and X refers to ACTH secretion elicited by the control stimulus in the presence of dexamethasone or corticosterone.

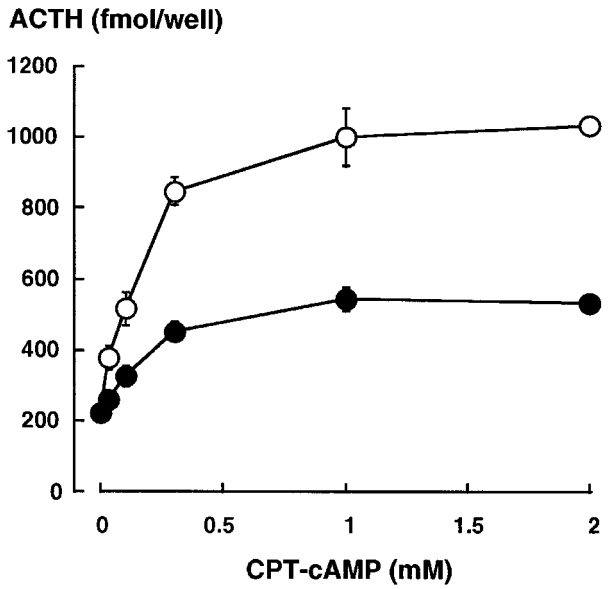

Figure 1 The effect of CPT-cAMP on ACTH secretion by AtT20 cells pretreated for 2 h with vehicle (○) or 100 nM dexamethasone (●). Means, the bars indicate s.e.mean, $n=5$/group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of four identical experiments.

Results

Experiments with AtT20 cells

Response to CPT-cAMP and dexamethasone Concentration-dependent stimulation of ACTH release was evoked by CPT-cAMP (Figure 1) and the response plateaued at 1 mM CPT-cAMP. Pretreatment with 100 nM dexamethasone significantly reduced the increase in CPT-cAMP-stimulated ACTH secretion, basal hormone output remained unaltered (Figure 1).

Effects of depolarizing agents The ACTH response to 1 mM CPT-cAMP could be enhanced further by the application of

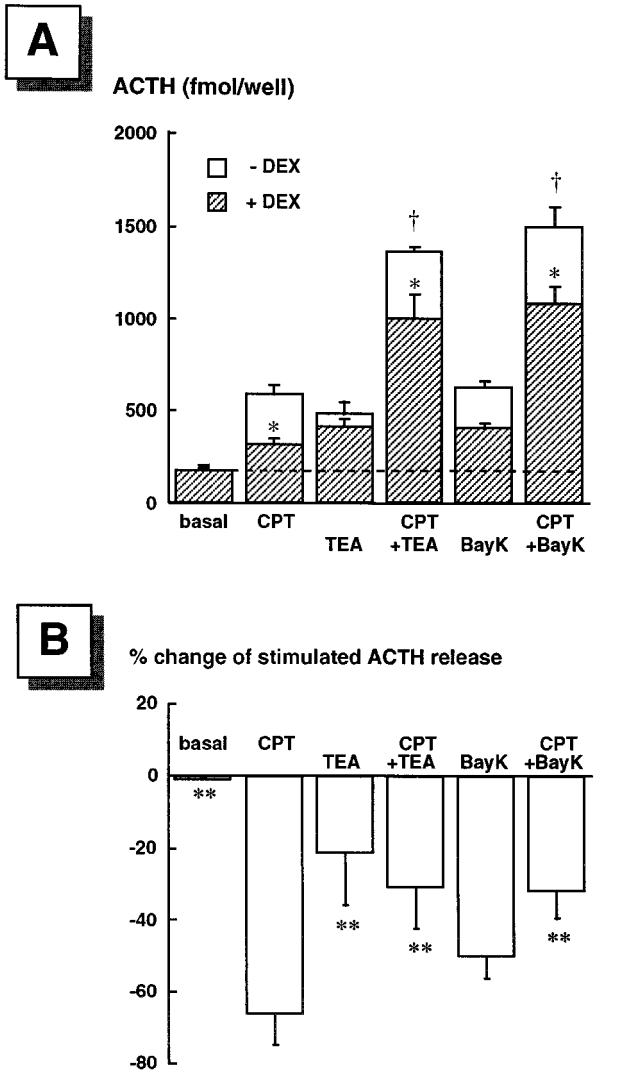

Figure 2 Effect of membrane depolarizing agents, 5 μM (−)BayK8644 (BayK) and 5 mM tetraethylammonium (TEA) on ACTH secretion elicited by 1 mM CPT-cAMP (CPT) after 2 h preincubation with vehicle or 100 nM dexamethasone in AtT20 cells. Data are means, bars indicate s.e.mean, $n=4$/group. (A) The empty columns show ACTH release by vehicle treated cells and the hatched columns show release after treatment with dexamethasone. †$P<0.02$ for the interaction between CPT alone and the respective depolarizing agents alone, 2-way ANOVA. *$P<0.05$ when compared to corresponding vehicle-treated release, 1-way ANOVA followed by contrast of means. (B) data are expressed as % change in the amount of ACTH secretion caused by dexamethasone when compared with the respective control groups shown in A. **$P<0.05$ when compared with CPT alone. 1-way-ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

5 mM tetraethylammonium (TEA) or the Ca^{2+}-channel activator (−)BayK8644 at 5 μM (Figure 2A). Both of these compounds stimulated ACTH release when given alone and exhibited synergistic interaction with CPT-cAMP. Similar data were obtained with another L-channel activator (+)202−791 at 5 μM (not shown).

The release of ACTH evoked by 1 mM CPT-cAMP was inhibited by 65.8±8.9% ($n=5$) by 100 nM dexamethasone, while basal secretion was not significantly altered (Figure 2A and B). The inhibition of ACTH secretion stimulated by a combination of 1 mM CPT-cAMP and 5 mM TEA or 1 mM CPT-cAMP and 5 μM (−)BayK8644 was significantly attenuated to 30.7±11.8% and 31.9±7.6% ($P<0.05$, $n=5$), respectively (Figure 2A and B). The combination of 1 mM CPT-cAMP with 5 μM (+)202−791 also significantly reduced the inhibition of the stimulated ACTH secretion by 100 nM dexamethasone to 27±1.3% ($P<0.05$, $n=4$/group, 1-way-ANOVA, Dunnett's test).

As both (−)BayK8644 and TEA partially reversed dexamethasone inhibition of CPT-cAMP stimulated ACTH secretion, the combination of these compounds with CPT-cAMP was tested subsequently, using a submaximal concentration (0.1 mM) of CPT-cAMP as well as 1 mM CPT-cAMP. The ACTH secretion elicited by 0.1 mM CPT-cAMP was significantly potentiated by 5 μM (−)BayK8644 and 5 mM TEA given together (Table 1). CPT-cAMP-induced ACTH secretion was inhibited by dexamethasone (1 nM to 1 μM) in a concentration dependent manner by up to 84% (Table 1). The combination of 5 μM (−)BayK8644 and 5 mM TEA elicited a 3-fold increase in ACTH secretion over basal levels and this response was not inhibited by dexamethasone (Table 1). The ACTH response elicited by the combination of 0.1 mM CPT-cAMP, 5 μM (−)BayK8644 and 5 mM TEA was also fully resistant to inhibition by 1−1000 nM dexamethasone (Table 1).

Similar results were obtained by combining 1 mM CPT-cAMP with 5 mM TEA and 5 μM (−)BayK8644. A summary of these data (Figure 3) shows that the extent of resistance to glucocorticoid inhibition was independent of the amplitude of stimulus-evoked ACTH secretion. For instance, although the ACTH response elicited by 5 mM TEA or 1 mM CPT-cAMP alone were similar, the ACTH response to 5 mM TEA was much more resistant to dexamethasone inhibition than the ACTH response to 1 mM CPT-cAMP.

Taken together, these results are in agreement with previous studies of CRF-induced ACTH release in AtT20 cells, which have shown that early glucocorticoid inhibition is effectively antagonized by depolarization of the membrane potential (Woods *et al*., 1994) and that TEA sensitive K^+-channels, particularly BK-type channels underlie the inhibitory action of corticosteroids (Shipston *et al*., 1996).

Experiments with primary cultures of rat anterior pituitary cells

Response to CPT-cAMP The time-course of the ACTH response to CPT-cAMP was close to linear over 4 h (Figure 4A). The concentration-response relationship between CPT-cAMP and ACTH release was similar at 60, 120 and 240 min, in that 0.1 mM CPT-cAMP elicited close to maximal

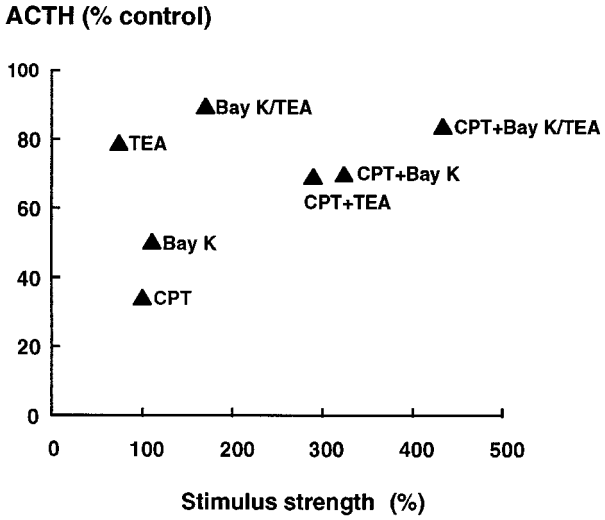

Figure 3 Size of the ACTH secretory response fails to predict the degree of dexamethasone inhibition in AtT20 cells. Data were plotted as stimulus strength *vs* ACTH response, where stimulus strength is the size of the evoked release of ACTH expressed as % of the response to 1 mM CPT-cAMP run in the same experiment, and ACTH response is the size of the ACTH response to the respective stimulus in cells preincubated with 100 nM dexamethasone, expressed as percentage of the control (i.e. no dexamethasone) response. Data are means $n=4$/group. Symbols: CPT, 1 mM CPT-cAMP; BayK, 5 μM (−)BayK8644; TEA, 5 mM tetraethylammonium; BayK/TEA, combination of 5 μM (−)BayK8644 and 5 mM tetraethylammonium; + sign indicates that two stimuli were applied together. Results shown are representative of two identical experiments.

Table 1 Characterization of the ACTH secretory response to CPT-cAMP, TEA and (−)BayK8644 in the presence of increasing dexamethasone concentrations in AtT20 cells

	Dexamethasone (nM)				
	0	*3*	*10*	*100*	*1000*
Vehicle	608±63				
0.1 mM CPT	1122±136	860±94	795±57	734±19	690±22
	(100±26%)	(49±18%)	(36±11%)	(25±4%)	(16±4%)
5 mM TEA +	1883±65	1973±125	1767±57	1783±84	1743±167
5 μM BayK	(100±5%)	(107±10%)†	(91±4%)†	(92±7%)†	(89±13%)†
0.1 mM CPT +	2919±93*	2912±126	2593±170	2626±62	3016±161
5 mM TEA +	(100±4%)	(100±5%)†	(86±7%)†	(87±3%)†	(104±7%)†
5 μM BayK					

Data show ACTH release expressed in fmol per well and represent means±s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±s.e.mean), as defined in Methods. *$P<0.02$ for the interaction between CPT and TEA/BayK in the absence of dexamethasone pretreatment, 2-way ANOVA. Statistical analysis of the effect of dexamethasone was carried out on the data expressed as percentages (values shown in parentheses) of the respective stimulus-evoked release, †$P<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

stimulation of ACTH release (Figure 4B). This was not the case at 30 min, where 1 mM CPT-cAMP was significantly more effective than 0.1 mM CPT-cAMP. All subsequent experiments were terminated at the 60 min time-point.

Lack of an effect of K^+-channel blockers and BayK8644 on corticosteroid inhibition of ACTH release Corticosterone (100 nM) reduced the ACTH response to 0.1 mM CPT-cAMP by $74.8 \pm 10.8\%$ ($n=5$) and this was not modified in the presence of TEA (5–20 mM) or (−)BayK8644 (5 μM). Similar findings were obtained with 30 nM dexamethasone. Combination of 5 mM TEA and (−)BayK8644 (5 μM) produced a significant enhancement of the ACTH releasing effect of CPT-cAMP (0.3 mM) (Basal: 34 ± 6 fmol/well; CPT-cAMP: 125 ± 5 fmol/well, $P<0.0001$, $n=6$; TEA + BayK: 60 ± 6 fmol/well, $P<0.0002$, $n=5$; CPT-cAMP + TEA + BayK: 204 ± 14 fmol/well, $P<0.03$, interaction, $n=7$, 2-way-ANOVA) but failed to modify the inhibition by 100 nM dexamethasone (% inhibition of CPT-cAMP *vs* CPT-cAMP + TEA + BayK: $63 \pm 7\%$ *vs* $66 \pm 6\%$, $n=4$). Finally, preincubation with charybdotoxin (30 nM) or apamin (100 nM) and the combination of these agents with 0.1 mM CPT-cAMP also failed to modify corticosterone inhibition of stimulated ACTH secretion (not shown).

These data indicate that in contrast to AtT20 cells, TEA-sensitive ion-channels are not fundamentally important for corticosteroid inhibition of ACTH secretion in primary cultures of rat anterior pituitary cells.

Depolarization with 40 mM KCl counteracts glucocorticoid effect on ACTH The ACTH responses to 0.1 mM CPT-cAMP and 40 mM KCl are shown in (Table 2). KCl (40 mM) synergized with 0.1 mM CPT-cAMP to stimulate ACTH release, furthermore, it produced a marked reduction in the inhibitory effect of dexamethasone (Table 2). Omission of Ca^{2+} from the incubation medium completely blocked ACTH release induced by CPT-cAMP alone or CPT-cAMP and KCl (not shown). Dexamethasone (10 nM) inhibited the ACTH response to 0.1 mM CPT-cAMP and 40 mM KCl by only $44 \pm 12\%$ ($n=4$), while that evoked by 0.1 mM CPT-cAMP was completely blocked. Similar results were also obtained using the naturally occurring corticosteroid corticosterone (Table 2).

The data above demonstrate that depolarization of the membrane potential markedly reduces the early inhibitory effect of glucocorticoids. However, the lack of a complete block of steroid action by 40 mM KCl raises the question of whether or not the inhibitory effect may be completely accounted for by the rapid induction of mRNA and protein by glucocorticoids.

Blockers of mRNA and protein synthesis abolish glucocorticoid inhibition The inhibition of 0.1 mM CPT-cAMP stimulated ACTH secretion by 10 nM dexamethasone was eliminated in the presence of 5,6-dichloro-furanosyl-benzimidazole riboside (DRB), an adenosine analogue and inhibitor of heteronuclear RNA synthesis (Egyházi *et al*., 1982) (Figure 5A). In the presence 40 mM KCl, DRB suppressed ACTH secretion elicited by 0.1 mM CPT-cAMP but no further change was observed after treatment with dexamethasone (Figure 5B). Therefore, the residual inhibition by corticosteroids observed in the presence of 40 mM KCl and 0.1 mM CPT-cAMP is likely to be due to glucocorticoid blockade of this DRB sensitive mechanism. Similar results were obtained using 0.1 mM puromycin, an inhibitor of protein synthesis (not shown).

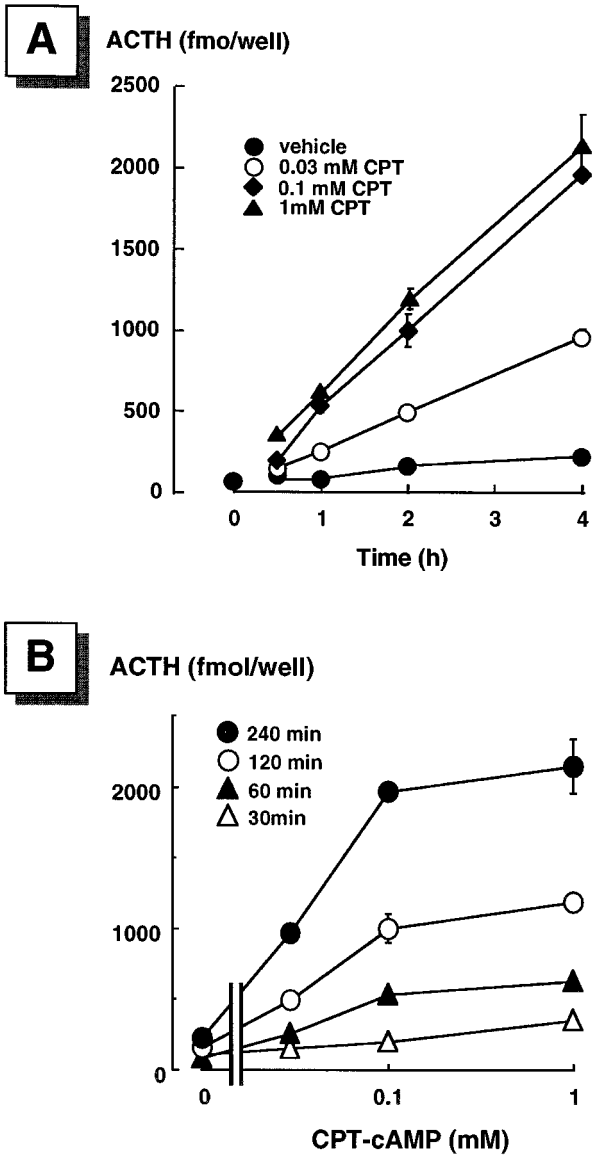

Figure 4 Effect of CPT-cAMP on ACTH secretion by primary cultures of rat anterior pituitary cells. (A) Time-course of hormone release in cells treated with vehicle (●), 30 μM CPT-cAMP (○), 0.1 mM CPT-cAMP (◆) or 1 mM CPT-cAMP (▲). (B) Concentration-dependent stimulation of ACTH secretion by CPT-cAMP at 30 min (△), 1 h (▲), 2 h (○) and 4 h (●) of incubation. Means, bars indicate s.e.mean, $n=4$/group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of two identical experiments.

Discussion

The data presented here show that control of the membrane potential is essential for glucocorticoid inhibition of stimulated ACTH release in both AtT20 cells and primary cultures of rat anterior pituitary cells. However, in contrast to tumoural AtT20 cells where BK-channels are pivotal targets of glucocorticoid action (Shipston *et al*., 1996), blockage of BK- and other TEA-sensitive K^+-channels failed to modify the inhibitory action of glucocorticoids in normal rat pituitary cells. Thus the data conform with the general hypothesis that glucocorticoids modulate the function of plasma membrane ion channels to reduce cellular responses of excitable cells (Joëls & de Kloet, 1994), and indicate that glucocorticoid-

Table 2 Counteraction of glucocorticoid inhibition of the ACTH secretory response by 40 mM KCl in primary cultures of rat anterior pituitary cells

	Corticosterone (nM)				
	0	*3*	*10*	*100*	*1000*
Vehicle	100.4±11.0				
0.1 mM CPT	163.8±6.9 (100±11%)	150.8±8.1 (80±13%)	132.6±15.2 (51±24%)	86.4±2.1 (−22±3%)	80.3±3.0 (−31±5%)
40 mM KCl	125.5±2.9				
0.1 mM CPT + 40 mM KCl	284.8±4.1* (100±2%)	269.8±16.3 (92±9%)	260.8±7.6 (87±4%)†	221.9±23.1 (66±13%)†	216.3±22.7 (63±12%)
	Dexamethasne (nM)				
	0	*3*	*10*	*100*	
Vehicle	66.1±7.0				
0.1 mM CPT	112.8±5.3 (100±11%)	62.8±2.5 (−7±5%)	54.5±3.3 (−25±7%)	52.0±3.1 (−30±7%)	
40 mM KCl	127.6±14.4				
0.1 mM CPT + 40 mM KCl	280.6±27.2** (100±13%)	192±31.8 (59±15%)†	187.2±26.1 (56±12%)†	165.2±11.8 (46±6%)†	

Data show ACTH release expressed in fmol per well and represent means±s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean±s.e.mean), as defined in Methods. $*P<0.0001$ and $**P<0.004$ for the interaction between CPT-cAMP and KCl in the absence of glucocorticoid pretreatment, 2-way ANOVA. Statistical analysis of the effect of glucocorticoids was carried out on the data expressed as percentages (values shown in the parentheses) of the respective stimulus-evoked release, $\dagger P<0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by contrast of means. Results shown are representative of two and three identical experiments for the corticosterone and dexamethasone dose responses respectively.

induced proteins may regulate the function of multiple plasma membrane ion channels.

Characteristics of the primary culture system as a model for secretagogue-glucocorticoid interaction and the use of CPT-cAMP

Previous studies have established that the main hallmarks of early glucocorticoid inhibition are: (i) selectivity for stimulated ACTH release, (ii) mediation through Type II glucocorticoid receptors, (iii) manifestation within 2 h, and (iv) a requirement for new mRNA and protein synthesis reviewed in (Shipston, 1995). These features have been largely retained in the AtT20 D16:16 cell line (Antoni, 1996), and also characterize the rat anterior pituitary primary culture system (Abou-Samra *et al*., 1986, and present study).

A further important property of the primary culture system is its responsiveness to physiological concentrations of CRF and AVP (Vale *et al*., 1983; Oki *et al*., (1990)). A somewhat surprising feature is the low response to depolarizing stimuli such as 40 mM KCl and BayK8644 given alone. These stimuli have been reported as inducers of robust ACTH release by acutely prepared pituitary cells (Gillies & Lowry, 1978; Antoni & Dayanithi, 1990b), tissue segments (Taylor *et al*., 1993) as well as AtT20 cells (Guild & Reisine, 1987). A plausible explanation of this finding is that the aforementioned preparations possess spontaneous electrical activity, which may be due to endogenous pacemaker potentials (e.g. AtT20 cells), or is derived from factors released upon tissue injury due to dissection in pituitary segments (Taylor *et al*., 1993), or is a remnant of the trophic actions of CRF *in vivo* (Ixart *et al*., 1991). Spontaneous electrical activity will facilitate the effects of the usage-dependent L-channel activator (−)BayK8644 (Sanguinetti & Kass, 1984). Furthermore, the secretagogue potencies of (−)BayK8644 as well as high KCl are likely to depend on tonic cyclic AMP-dependent phosphorylation which is required for the optimal functioning of L-type Ca^{2+} channels (Armstrong & Eckert, 1987) and exocytotic processes (Morgan *et al*., 1993).

With respect to the requirement for cyclic AMP, it is known that acutely dispersed cells produce much more reliable responses to short pulses CRF if 'primed' with several pulses of the peptide (Antoni *et al*., 1990b). Moreover, it has been reported that cyclic AMP and Ca^{2+} synergize to trigger ACTH release from permeabilized AtT20 cells (Guild, 1991), further indicating that cyclic AMP-dependent processes acting in parallel to the mobilization of Ca^{2+} are important for the secretory response of corticotroph cells. It is of note that while depolarization with 40 mM KCl was not a reliable stimulus of ACTH release on its own, it consistently produced a synergistic enhancement of the CPT-cAMP induced ACTH response. As the main result of KCl depolarization is Ca^{2+} influx through voltage-operated channels (Meier *et al*., 1988), the synergy of CPT-cAMP and KCl depolarization appears analogous to the Ca^{2+} potentiation of cAMP-induced ACTH release in AtT20 cells. Similar results have been obtained using physiological concentrations of CRF (Lim *et al*., unpublished).

A cell- membrane permeant, non-metabolizable analogue of cyclic AMP, CPT-cAMP, was used as the secretagogue stimulus in this study in order to produce a sustained increase in cyclic AMP-dependent phosphorylation. As the physiological ACTH secretagogues, CRF or CRF and AVP in combination, activate cyclic AMP-dependent phosphorylation in corticotrophs (see Antoni, 1993), CPT-cAMP will mimic this aspect of agonist action. It is also clear that CRF-induced cyclic AMP responses are subject to negative feedback regulation by intracellular free Ca^{2+} at the level of adenylyl cyclase and cyclic AMP hydrolysis in AtT20 cells (Antoni, 1996) as well as rat anterior pituitary cells (Antoni, 1997). CPT-cAMP is largely resistant to Ca^{2+}-feedback and this property makes it a potentially useful tool for studying processes of ACTH release downstream of cyclic AMP formation. It is of note, however, that CRF elicits oscillations of intracellular free Ca^{2+} in AtT20 cells as well as normal rat corticotrophs, (see Antoni, 1993 for review), which may be accompanied by oscillations of intracellular cyclic AMP levels (Cooper *et al*., 1998). Thus, clamping the intracellular cyclic AMP through CPT-cAMP, which leads to sustained increases of intracellular free Ca^{2+} as opposed to oscillations (Mollard

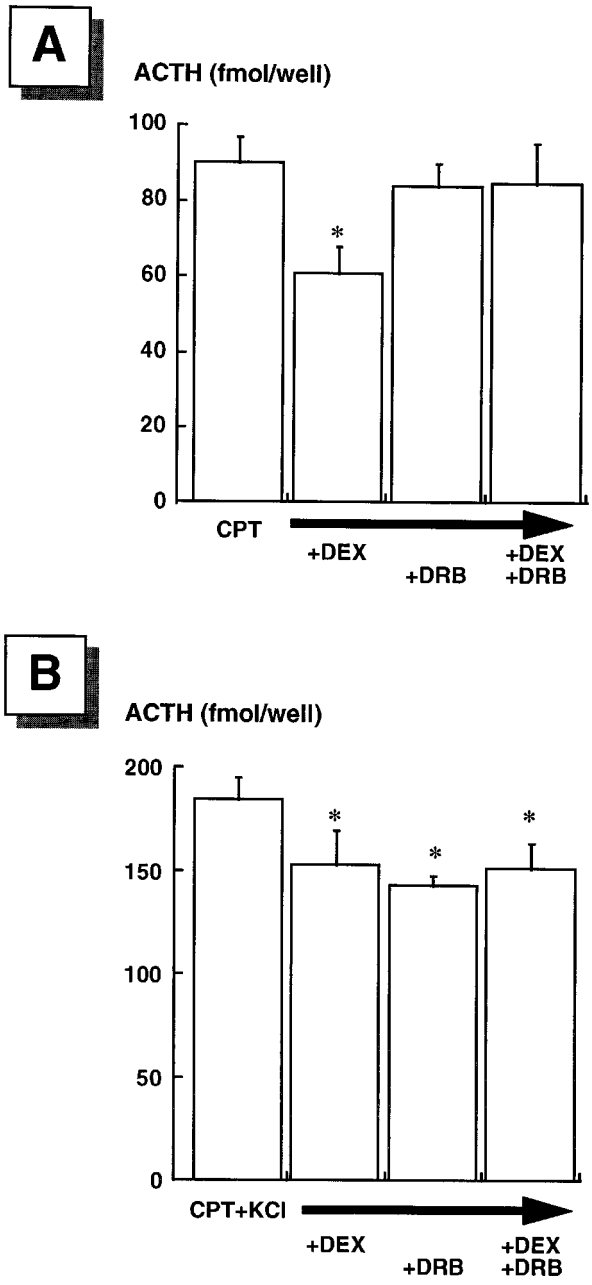

Figure 5 Effect of the RNA synthesis inhibitor 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) on ACTH secretion in primary cultures of rat anterior pituitary cells. 100 μM DRB and 100 nM dexamethasone (DEX) were applied 2 h before challenging the cells with (A) 0.1 mM CPT-cAMP or (B) 0.1 mM CPT-cAMP and 40 mM KCl for 60 min. Basal ACTH release was 44 ± 3 fmol/well and was not altered by DRB or dexamethasone. Data are means, bars indicate s.e.mean, $n = 5$/group. $^*P < 0.05$ when compared with control group receiving vehicle only. 1-way-ANOVA followed by Newman-Keuls test. Results shown are representative of four identical experiments.

et al., 1992), may potentially alter some properties of the ACTH response when compared with physiological agonists. Indeed, inhibition of mRNA or protein synthesis significantly attenuated the release of ACTH by rat pituitary corticotrophs to CPT-cAMP-based stimuli, whereas such manipulations were without effect on CRF-induced ACTH secretion (Dayanithi & Antoni, 1989; Taylor *et al.*, 1993 and Lim *et al.*, unpublished). In other respects, however, CPT-cAMP-stimulated ACTH release was largely dependent on extracellular Ca^{2+} and blocked by glucocorticoids similarly to ACTH secretion evoked by CRF.

Properties of early glucocorticoid inhibition in AtT20 cells

The results with AtT20 cells extend previous work from this laboratory examining CRF-induced ACTH release in these cells. Overall, ACTH release induced by CPT-cAMP alone was potently inhibited by dexamethasone. By contrast, responses to stimuli that cause depolarization such as TEA, (−)BayK8644 or (+)202−791 were resistant to glucocorticoid inhibition. It was also clear that the nature of the stimulus rather than the size of the evoked response predicted the degree of glucocorticoid inhibition in AtT20 cells. Thus, these data are in full agreement with earlier findings from this laboratory indicating that the principal target of corticosteroid inhibition is the membrane potential (Pennington *et al.*, 1994; Shipston *et al.*, 1996) rather than the secretory apparatus as proposed by others (Castellino *et al.*, 1992). Further, the data are in agreement with the findings that BK-channels (Shipston *et al.*, 1996) are pivotal for glucocorticoid inhibition in AtT20 cells.

Corticosterone action in primary cultures: deviation from and convergence to AtT20 model

Importantly, the involvement of plasma membrane ion-channels in glucocorticoid action has not been previously addressed in normal rat corticotrophs. A report from another laboratory has shown (Halili-Manabat *et al.*, 1995) that ACTH secretion stimulated by the depolarizing agent veratridine, an opener of tetrodotoxin sensitive Na^+-channels is resistant to inhibition by dexamethasone, and our group has reported similar findings in preliminary form using combinations of CRF and veratridine (Antoni & Woods, 1992). However, no stimuli physiological to corticotrophs have been identified so far that operate through Na^+-channels and thus counteract glucocorticoid feedback inhibition (Antoni, 1993; Halili-Manabat *et al.*, 1995).

In sharp contrast to AtT20 cells, concentrations of TEA as high as 20 mM failed to dent significantly the early inhibitory action of corticosterone or dexamethasone in primary cultures of rat anterior pituitary cells. Also of note is that apamin and charybdotoxin, blockers of SK- and BK-type Ca^{2+}-activated K^+-channels were also without effect. In contrast to the channel blockers tested so far, 40 mM KCl synergized with CPT-cAMP to stimulate the release of ACTH, while having relatively little effect on its own. Furthermore, the early inhibitory effects of dexamethasone as well as corticosterone were drastically reduced with CPT-cAMP and KCl as the stimulus. The release of ACTH under these conditions was fully dependent on extracellular Ca^{2+}, hence indicating that the mechanism of hormone release is not fundamentally altered by the addition of 40 mM KCl.

Overall, these findings are surprising, as channels sensitive to the K^+-channel blockers applied in the present study, and functionally relevant for the regulation of ACTH release have been previously reported in various preparations of corticotroph cells (Marchetti *et al.*, 1987; Antoni & Dayanithi, 1990a). The possibility that the K^+-channel profile of corticotrophs is altered by culture conditions cannot be discounted at present. A recent report (Lee & Tse, 1997) suggests that CRF-induced activation of cultured rat pituitary corticotrophs is the result of the inhibition of a K^+-current that is insensitive to TEA as well as 4-aminopyridine. However, as noted above, the role of K^+

Table 3 Comparison of early corticosteroid inhibition of stimulated ACTH release in AtT20 cells and primary cultures of rat anterior pituitary cells

Common features:
Onset within 2 h
Mediated by Type II glucocorticoid receptor
Requirement of new mRNA and protein synthesis
Specific for agonist-induced release
Opposed by depolarization of the membrane potential
Specific features:
AtT20 cells
Mediated by BK-type potassium channels
Primary cultures
Mediated by unidentified ion channel
May involve transcriptional block of protein expression required for sustained release of ACTH

channels in glucocorticoid feedback has not been previously examined in non-tumoural corticotrophs.

The nature of the residual inhibitory effect of glucocorticoids on KCl/CPT-cAMP stimulated ACTH release was revealed in experiments with inhibitors of protein and mRNA synthesis. As some reports suggested that early corticosteroid inhibition does not require gene induction (Taylor *et al*., 1993) we have re-examined this issue in the primary culture system. Similarly to actinomycin D (Arimura *et al*., 1969; Portanova & Sayers, 1974; Dayanithi *et al*., 1989) DRB blocked the early corticosteroid inhibition of ACTH release, indicating a requirement for mRNA synthesis. Moreover, DRB as well as puromycin caused similar reductions in ACTH release induced by 40 mM KCl/0.1 mM CPT-cAMP in the presence of 100 nM dexamethasone, which had no additional effect in the presence of these agents. The findings indicate that in case of relatively high (>4 fold basal) ACTH responses elicited by CPT-cAMP-based stimuli the ACTH secreted is derived through the synthesis of new mRNA and protein, plausibly POMC, which is blocked by glucocorticoids. In contrast to CPT-cAMP-based stimuli, there was no apparent requirement for mRNA or protein synthesis when CRF was used to stimulate ACTH release (Abou-Samra *et al*., 1986; Dayanithi *et al*., 1989; Taylor *et al*., 1993). Thus oscillating Ca^{2+}/cAMP signals may be more efficient in mobilizing/processing ACTH without a requirement for *de novo* protein synthesis than CPT-cAMP which elicits prolonged elevations of intracellular Ca^{2+} lacking an oscillatory pattern (Mollard *et al*., 1992).

In summary (Table 3), it is intriguing that the same physiologically relevant phenotype of early corticosteroid inhibition is found in AtT20 cells and rat corticotrophs, including counteraction by membrane depolarization, but the underlying membrane-control mechanisms have strikingly distinct pharmacology. Further studies should clarify whether glucocorticoids are capable of inducing different families of ion-channel subunits depending on the cell system under study (Attardi *et al*., 1993; Levitan *et al*., 1996) or evoke the synthesis of proteins that regulate the activity of several distinct ion channels through generic mechanisms such as protein phosphorylation (Wang *et al*., 1997). Data from our laboratories (Shipston *et al*., 1996) favour the latter possibility in the case of adenohypophysial corticotrophs.

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