Pergamon

0960-0760(93)E0032-3

J. Steroid Biochem. Molec. Biol. Vol. 48, No. 4, pp. 337–345, 1994

0960-0760/94 $6.00 + 0.00

Calcium and Protein Kinase C Regulation of the Glucocorticoid Receptor in Mouse Corticotrope Tumor Cells

Karen E. Sheppard

Molecular Physiology Laboratory, Baker Medical Research Institute, Prahran, Vic. 3181, Australia

The effect of increasing intracellular free calcium and activating protein kinase C on glucocorticoid receptor (GR) expression was investigated in AtT-20 cells, a mouse corticotrope tumor cell line. Treatment of AtT-20 cells with the calcium ionophore A23187 induced a rapid time- and dose-dependent decrease in [^{3}H]dexamethasone ([^{3}H]DEX) binding when measured in intact cells. Binding fell to 16% of control following 3 h of treatment with 10 μM A23187. In contrast, A23187 did not acutely effect steady state levels of GR mRNA, although levels fell to 76 $\pm$ 1% of control after 8–15 h of treatment. Scatchard analysis of A23187 treated cultures demonstrated a decrease in GR binding capacity but no change in affinity for [^{3}H]DEX. Acute inhibition of protein synthesis with cycloheximide had no effect on [^{3}H]DEX binding, suggesting that the calcium-dependent decrease was not simply due to inhibition of GR protein synthesis. In contrast to the A23187 induced decrease in [^{3}H]DEX binding in intact cells, when binding was measured in cytosol extract from A23187 treated cultures there was no decrease. These data suggest that the A23187 induced decrease in GR binding in whole cells is not due to a decrease in GR protein but reversible conversion of the receptor to a non-binding form. Inducing calcium influx only through L-type voltage-dependent calcium channels with BAY K8644 also decreased [^{3}H]DEX binding at AtT-20 cells, though the effect was less than that induced by A23187. Although activation of protein kinase C with phorbol ester transiently increases intracellular free calcium in AtT-20 cells, when cells were treated for 0.5 to 22 h with phorbol 12-myristate 13-acetate, there was no acute fall in [^{3}H]DEX binding, and only a small decrease following 16 h of treatment. These data demonstrate that sustained increases in intracellular calcium in corticotropes can induce a rapid and marked decrease in GR binding. The mechanism is post-translational and involves the reversible conversion of the receptor to a non-binding form. In addition, the cellular milieu is clearly important in conferring non-binding status on GR since once the cell is disrupted GR binding is restored.

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INTRODUCTION

The glucocorticoid receptor (GR) is a ligand-activated nuclear transcription factor. In its non-activated state it is found in the cytoplasm, and upon ligand binding a series of events takes place leading to translocation of the receptor–ligand complex to the nucleus where it regulates gene transcription. By altering the level of functional GR the cellular glucocorticoid response can be modified [1–5]. Many groups have demonstrated down-regulation of GR expression by ligand; the mechanism(s) involved occur in a cell specific manner and include decreases in GR gene transcription [6, 7], GR mRNA [8–10] and the half-life of GR protein [7, 10, 11]. Glucocorticoid induced up-regulation of GR has also been reported [12–15]; although the mechanisms involved are unknown, the glucocorticoid effect is probably not a direct genomic one, considering that a complete glucocorticoid response element sequence is absent within a 3 kilobase 5′ flanking sequence of the GR gene [16]. In addition to ligand-induced changes in GR expression, various neurotransmitters [17–19], other steroids [20] and cyclic nucleotides [21–24] have also been reported to regulate cellular levels of GR in a variety of cell types.

Most hormone receptors rely on second messenger systems to relay their intracellular signal. Classical steroid receptors are unusual in that they are localized in either the cytoplasm or the nucleus of a cell, and are themselves part of their intracellular signal transduction pathway. Thus, in addition to down-regulation of GR, interactions between the receptor and various

Received 2 Apr. 1993; accepted 9 Nov. 1993.

intracellular pathways can potentially play an important role in the overall cellular response to glucocorticoids. Interactions between the phospholipid- and cAMP-dependent pathways have been extensively reported in many cells [25–28], though little is known about the possibility of cross-talk between GR and other intracellular signal pathways. Recently the transcription factors c-Fos and c-Jun, which are the end point of many signal transduction pathways, were shown to associate with GR and inhibit its action [29–31]. In addition, the oncogene product of v-*mos* was demonstrated to impair glucocortioid function by affecting nuclear retention and reutilization of the receptor [32]. Furthermore, rapid inhibition of the glucocorticoid response by corticotropin-releasing factor (CRF) and adenylate cyclase/protein kinase A (PKA) pathway has been demonstrated in corticotropes [33, 34]. The inability to demonstrate a rapid CRF-induced decrease in GR binding [24] suggests that GR has not been down-regulated by CRF but has been converted into a non-functional form.

The aim of the present study is to determine the possible interaction between GR and intracellular pathways that are involved in regulating expression of POMC in AtT-20 cells, a mouse corticotrope tumor cell line. In these cells POMC gene transcription and/or ACTH release are inhibited by glucocorticoids, and stimulated by CRF through the adenylate cyclase/PKA pathway, arginine vasopressin (AVP) through phospholipid/protein kinase C (PKC) pathway, and various factors that increase intracellular free calcium (for reviews see [35–39]). We have previously demonstrated a CRF- and cAMP-mediated decrease in GR biosynthesis in AtT-20 cells [24]. The present study explores the potential regulation of AtT-20 GR expression by PKC activation and intracellular calcium. We report that increasing intracellular free calcium either with a calcium ionophore or more specifically through voltage-dependent calcium channels rapidly decreases the level of GR binding in AtT-20 cells; in contrast, activation of PKC with phorbol ester has little effect on GR expression in these cells.

EXPERIMENTAL

Cell culture and treatment

AtT-$20_{D16\text{-}16}$ mouse corticotrope tumor cells were grown at 37°C under 95% O_2 and 5% CO_2 on 60 mm tissue culture plates in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal calf serum (FCS), 5% horse serum (HS), penicillin (100 U/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). Cells were treated and harvested 4–7 days after plating, and serum starved 16–24 h prior to treatment. A23187 (10 mM stock in Me_2SO), BAY K8644 (10 mM stock in methanol), phorbol 12-myristate 13-acetate (PMA: 10 μM stock in 0.2% Me_2SO), or cycloheximide (10 mM stock in water) were added directly to media and the same volume of Me_2SO or methanol added to the respective control. At the end of the incubation period cells were collected by scraping into DMEM, centrifuged (200*g* for 7 min) and then resuspended in DMEM or TMD buffer (10 mM Tris, 100 mM $NaMoO_4$, 1 mM dithiothreitol, pH 7.4) for determination of GR binding capacity, or AT buffer (10 mM Tris, pH 8; 3 mM $CaCl_2$; 2 mM $MgCl_2$; 0.5 mM dithiothreitol; 0.15% Triton X-100) containing 0.3 M sucrose for RNA extraction. Media were collected from cultures prior to cell harvesting and analyzed for immunoreactive β-endorphin by a previously described RIA [40]. For all assays, each plate represents a single determination, thus "*n*" represents the number of plates per data point. All data are expressed as mean ± SEM.

Preparation of cells for [^{3}H]dexamethasone ([^{3}H]DEX) binding assays

For intact cell and cytosol extract binding assays cells were collected into DMEM and pelleted by centrifugation at 200*g* for 7 min. Cell pellets were resuspended in DMEM for intact cell binding assays and in TMD buffer for cytosol extract binding. In all binding assays GR levels were quantified by a single saturating dose of [^{3}H]DEX (30 nM), and non-specific binding was determined in the presence of a 200-fold excess of unlabeled DEX. For Scatchard analysis concentrations of [^{3}H]DEX ranged over dilutions from 28 to 1.7 nM and non-specific binding was determined at each dose of tracer.

[^{3}H]DEX binding in intact cells

Cell pellets resuspended in DMEM were added to pre-gassed (5% CO_2, 95% O_2) glass tubes containing [^{3}H]DEX with or without a 200-fold excess of DEX. Tubes were then covered in parafilm and incubated at room temperature for 60 min at which time receptor binding had reached equilibrium (data not shown). Following the incubation 3 ml of ice cold DMEM was added and the cells pelleted by centrifugation (200*g* for 5 min at 4°C); this was repeated 3 times.

For Scatchard analysis, washed [^{3}H]DEX labeled cells were taken up in a total volume of 1.2 ml of 1.0 M perchloric acid and placed in glass scintillation vials. DNA was hydrolyzed by incubating vials at 68°C for 30 min, after which 0.2 ml of the solubilized nucleotides were taken and quantified by the diphenylamine assay [41] and the remaining 1.0 ml counted. Herring sperm DNA was used for a standard curve and specific [^{3}H]DEX binding was then normalized to DNA recovered.

To determine [^{3}H]DEX binding in the nuclei and cytoplasm of [^{3}H]DEX labeled cells a nuclear/cytoplasmic binding assay adapted from Spelsberg *et al.* [42] was employed. Briefly, washed [^{3}H]DEX labeled cells were taken up into 0.5 ml of cold solution B [1.0 M sucrose, 10% (v/v) glycerol, 0.2% (v/v) Triton X-100, 10 mM KCl and 50 mM Tris, pH 7.4], layered over 0.3 ml of solution C [1.4 M sucrose, 10% (v/v) glycerol, 0.2% (v/v) Triton X-100, 10 mM KCl and 50 mM Tris, pH 7.4] and centrifuged for 20 min at

6000*g* and 4°C. The supernatant was either discarded, or collected and added to 15 ml of scintillation fluid for the determination of cytoplasmic binding. The nuclear pellet was taken up in solution D [10% (v/v) glycerol and 50 mM Tris, pH 7.4] and placed on nitrocellulose filters (2.5 cm diameter, 0.45 μm pore size: Millipore) under vacuum. Filters were dried at 110°C for 10 min. Dried filters were placed in 20 ml scintillation vials containing 1.2 ml of 1.0 M perchloric acid. DNA was hydrolyzed and quantified as described for whole cell preparation. Scintillation fluid (15 ml) was added to the remaining 1 ml for counting.

[^{3}H]DEX binding in cytosol extract

Cells resuspended in TMD buffer were homogenized by hand (Teflon–glass homogenizer) and the homogenate centrifuged (105,000*g* for 40 min at 4°C) to yield cytosol. Cytosol extracts (150 μl) were incubated (25°C for 90 min) with 50 μl of TMD buffer containing [^{3}H]DEX with or without DEX (200-fold excess). To separate receptor-bound from free steroid, an equal volume of equilibrated hydroxylapatite slurry was added to each tube and shaken periodically for 30 min at 4°C. The mixture was then centrifuged (1000*g* for 3 min at 4°C), supernatant decanted and the hydroxylapatite pellet resuspended in 3 ml of wash buffer (1% Tween 80, 5 mM Na_2HPO_4, 10 mM Tris and 1.5 mM Na_2-EDTA, pH 7.4, 4°C). This sequence was repeated 3 times. Washed hydroxylapatite pellets were extracted with 2.0 ml ethanol at room temperature for 30 min, centrifuged (1000*g* for 5 min) and the supernatant added directly to 10 ml of scintillation fluid and counted. A sample of the cytosol was taken and protein determined by the Bradford method [43]. For determination of DNA, the ultracentrifuge pellet was resuspended in 1.7 ml TE buffer (10 mM Tris and 1.0 mM Na_2-EDTA pH 7.4, 4°C), perchloric acid (7 M) was then added to give a final concentration of 1 M. DNA was hydrolyzed by incubating at 68°C for 30 min, after which the tubes were centrifuged to removed debris and the solubilized nucleotides were taken and quantified by the diphenylamine assay [41]. Cytosol extract binding was then normalized to cytosolic protein. Normalization of cytosol extract binding to DNA reflected the same results observed when binding was normalized to protein (data not shown).

Cellular protein determination

Following 3 h treatment with A23187 (1 μM), cells were collected into TE buffer containing 1% Triton-X, vortexed, freeze–thawed twice, and then centrifuged (18,000*g* for 5 min). The supernatant was then assayed for protein by the Lowry method [44].

RNA isolation and quantification

Following treatment cells were pelleted and resuspended in ice-cold AT buffer containing 0.3 M sucrose. Cytoplasmic RNA was then isolated as described previously [24]. Briefly, nuclei were separated from the cytoplasmic fraction by layering disrupted cells over AT buffer containing 0.4 M sucrose and centrifuging at 900*g* for 10 min. The cytoplasmic fraction (supernatant) was then proteinase K digested, phenol/chloroform extracted, and cytoplasmic RNA precipitated with isopropanol. For cytoplasmic RNA quantification, samples were reconstituted in TE buffer and RNA concentration determined by absorbance at 260 nm.

Recombinant clones

All RNA probes were synthesized with the Promega transcription system (Riboprobe Gemini system II: Promega, WI), to a specific activity of $0.5–1.0 \times 10^9$ cpm/μg RNA. As described previously [24], a 216 basepair cDNA fragment corresponding to a portion of the DNA binding region of the mouse GR was used to synthesize both sense RNA and radiolabeled antisense RNA probes.

Solution hybridization/RNA protection assay

GR mRNA levels were quantified using a solution hybridization/S1 nuclease protection assay, described previously [14]. In brief, *in vitro* synthesized sense RNA and cytoplasmic RNA were hybridized to a ^{32}P-antisense RNA probe for 18 h at 65°C, followed by S1 nuclease digestion (500–700 U) for 60 min at 56°C. Samples were then phenol/chloroform extracted, isopropanol precipitated, reconstituted in TE buffer and run on a 5% non-denaturing polyacrylamide gel. Specific mRNA- and sense RNA-^{32}P-antisense RNA hybrid bands were excised from the dried gel and counted by liquid scintillation spectrophotometry. GR mRNA levels were determined by comparison to the standard curve and normalized to the total amount of cytoplasmic RNA loaded.

Statistics

Statistical analysis was by one-way analysis of variance followed by Fisher PLSD test. All data are expressed as mean $\pm$ SEM.

RESULTS

A23187 time course

AtT-20 cells were treated for various time periods with the calcium ionophore A23187 (1 μM). Following treatment the level of specific nuclear localized [^{3}H]DEX binding was determined by the nuclear/cytoplasmic binding assay and normalized to DNA recovered, or GR mRNA was measured and normalized to cytoplasmic RNA. As illustrated in Fig. 1(A), A23187 induced a rapid time-dependent decrease in [^{3}H]DEX binding which fell to $57 \pm 2\%$ of control by 3.5 h and remained at this level at the 7 h ($59 \pm 1\%$) and 14 h ($53 \pm 2\%$) time points. A slight decrease (10–14%) in the DNA recovered from A23187 treated cultures was observed at all time points [Fig. 1(B)]. GR mRNA levels decreased by $24\% \pm 1$ at 8 h and remained at this level at the 15 h time point [$22\% \pm 4$: Fig. 1(C)]. For all A23187 time points examined there

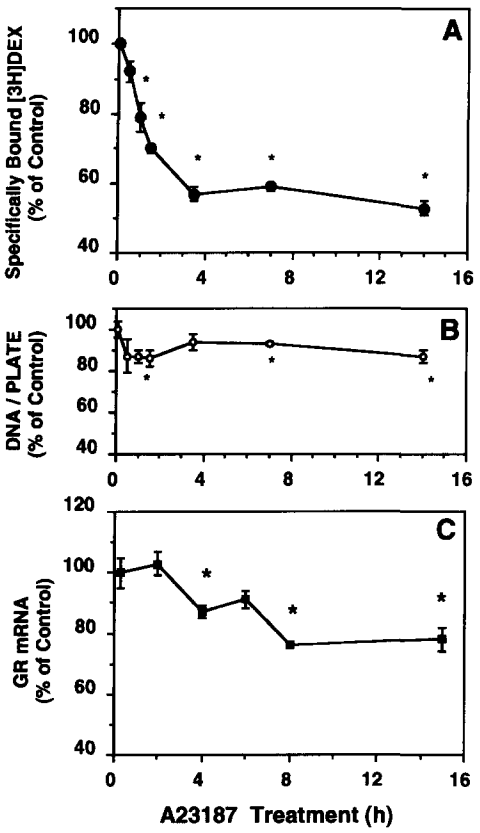

Fig. 1. Effect of A23187 on GR binding (A), DNA recovered (B), and GR mRNA levels (C) in AtT-20 cells. Cell cultures were incubated for the indicated times with 1.0 μM A23187. Following treatment, either [^{3}H]DEX nuclear localized binding was measured and normalized to total DNA recovered (A), or GR mRNA levels were determined and normalized to cytoplasmic RNA (C). At time zero, levels of GR binding, DNA recovered, and GR mRNA were taken as 100% and the remaining points presented as a percentage thereof. Each point represents the mean $\pm$ SEM; $n = 4$ plates per time point. *$P < 0.05$ compared with control.

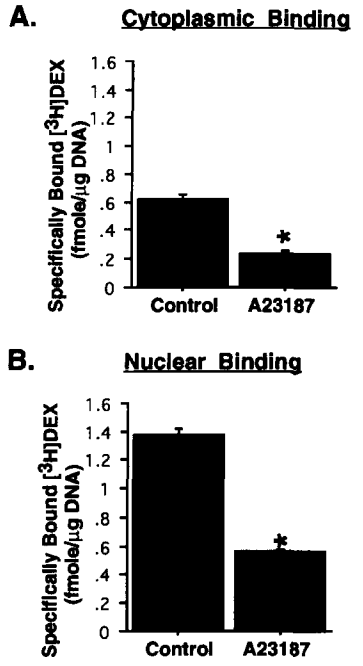

Fig. 2. Comparison of A23187-mediated decrease in GR binding in cytoplasm (A) and nuclei (B) of AtT-20 cells. At T-20 cells were treated with A23187 (3 μM) for 3 h, cells were then collected and incubated with [^{3}H]DEX $\pm$ DEX. Following the incubation specific binding was measured in the nucleus and cytoplasm as described in 'Experimental'. All data were normalized to the DNA recovered in the nuclear fraction. Each point represents mean $\pm$ SEM: $n = 5$: *$P < 0.05$.

was no consistent change in AtT-20 cytoplasmic RNA levels recovered (data not shown).

Nuclear and cytoplasmic localization of [^{3}H]DEX binding

Granted that non-specific binding in the cytoplasmic fraction is 50–70% of total cytoplasmic binding (compared with 5–15% in the nuclear fraction), binding in cytoplasm was not routinely measured. However, the A23187 induced decrease in binding observed in the nuclear fraction (41% of control) was also seen in the cytoplasm (39% of control: Fig. 2). The ratio of cytoplasmic to nuclear binding in control and A23187 treated cells was 1:2.2 and 1:2.3, respectively, suggesting that this treatment does not alter the ability of activated GR to translocate to the nucleus.

A23187 dose–response

To study the dose-related effect of A23187 on GR binding, AtT-20 cells were incubated for 3 h with a range of concentrations of A23187 from 0.1 to 10 μM. As depicted in Fig. 3(A), A23187 decreased nuclear [^{3}H]DEX binding in a dose-dependent manner. Significant inhibition by A23187 was observed at 1 μM, at which dose [^{3}H]DEX binding fell to 45 $\pm$ 2% of control and at the highest dose of A23187 (10 μM), [^{3}H]DEX binding was 16 $\pm$ 1% of control. As an

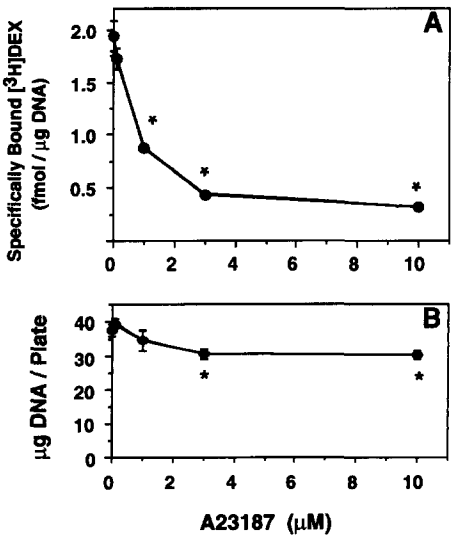

Fig. 3. A23187 Dose–response effect on GR binding (A) and DNA recovered (B) in AtT-20 cells. Cells were incubated for 3 h with the indicated concentrations of A23187. Following incubation, cells were collected and [^{3}H]DEX nuclear localized binding determined and normalized to DNA recovered. Each point represent the mean $\pm$ SEM; $n = 4$. *$P < 0.05$ compared to untreated cultures.

indication of the toxicity of the doses of A23187 the DNA recovered at each dose is plotted in Fig. 3(B). As seen in the time course experiment A23187 from 3 to 10 μM induced a small decrease in DNA recovered, suggesting that a small number of cells had detached from the tissue culture plate during A23187 treatment.

Scatchard analysis

The decrease in [^{3}H]DEX binding following A23187 treatment preceded and was greater than the time-dependent decrease in GR mRNA, suggesting that A23187 has a predominant post-transcriptional action on GR expression. To further dissect the possible mechanism by which calcium is decreasing GR binding in these cells, the affinity of GR for DEX and the number of binding sites were assessed by Scatchard analysis in A23187 treated (1 μM for 3 h) and non-treated cultures. As depicted in Fig. 4(B), in a whole cell binding assay the affinity of [^{3}H]DEX for GR is unchanged in A23187 treated cultures. In non-treated cultures: $K_d = 6.3$ nM and $B_{max} = 88$ fmol, and in A23187 treated; $K_d = 7.3$ nM and $B_{max} = 51$ fmol.

GR binding: comparision of cytosol extract to intact cells

Two other possible mechanisms by which A23187 could decrease GR binding are either by decreasing receptor number and/or converting the receptor to a non-binding form. To further examine the A23187 induced decrease [^{3}H]DEX binding observed in intact cells, binding was measured in a cytosol extract. AtT-20 cells were treated for 3 h with 3 μM A23187. Following treatment either cytosol extract was prepared which was then incubated with [^{3}H]DEX ± DEX and specific binding determined by the hydroxylapatite method, or intact cells were incubated with [^{3}H]DEX ± DEX and specific binding measured in the nuclei and cytoplasm by the nuclear/cytoplasmic assay. In contrast to the potent A23187 induced decrease in binding in both cytoplasm and nuclei (32 and 38% of control, respectively), in cytosol extract there was only a slight decrease in [^{3}H]DEX binding (Table 1). To verify that both types of binding assays (intact cell vs cytosol extract) can reflect the same result when there is a change in GR protein levels, GR binding in cytosol extract was measured in CRF treated and non-treated cells. CRF has been previously demonstrated to decrease GR binding in intact AtT-20 cells [24], this decrease being partly due to a decrease in GR mRNA and presumably GR protein. When [^{3}H]DEX binding was measured in cytosol extract from CRF treated cells (10 nM for 24 h) there was a 30% decrease in binding (data not shown). This decrease is similar in magnitude to that observed in intact cells (30–50%) following CRF treatment [24]. In addition differences in GR binding levels between various cell lines are similar in both types of binding assays (data not shown). These data suggest that the difference between the intact cell binding assay and binding in cytosol extract from A23187 treated cells is not a reflection of the different binding assays, but rather that in intact cells A23187 treatment converts the receptor to a non-binding form.

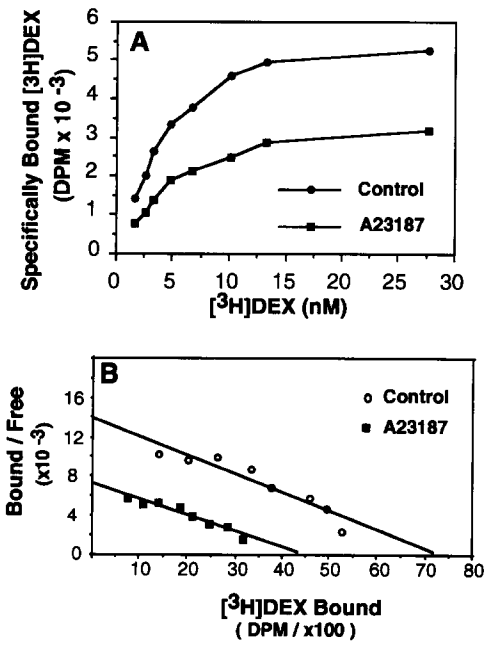

Fig. 4. Saturation curve (A) and Scatchard analysis (B) of [^{3}H]DEX binding in control and A23187 treated cultures. AtT-20 cells were incubated with 1.0 μM A23187 for 3 h. Following the incubation cells were removed and whole cell [^{3}H]DEX binding determined. The concentration of radioligand ranged from 1.7 to 28 nM and non-specific binding was determined at each dose of tracer. The binding site concentration (B_{max}), dissociation constant (K_d), and correlation coefficient (r) for control cultures are $B_{max} = 88$ fmol, $K_d = 6.3$ nM, and $r = 0.90$; and for A23187 treated cultures, $B_{max} = 51$ fmol, $K_d = 7.3$ nM, and $r = 0.93$.

Table 1. Comparison of [^{3}H]DEX binding in cytosol extract and intact cells following A23187 treatment

	[^{3}H]DEX binding		
		Whole cells	
	Cytosol extract	Nuclear	Cytoplasmic
Control	100%	100%	100%
A23187	91 ± 6%	32 ± 5%	38 ± 6%

AtT-20 cells were treated for 3 h with either A23187 (3 μM) or DMSO (Control). Following treatment cells were collected and either incubated with tracer and nuclear and cytoplasmic binding measured, or cytosol was prepared and this was then incubated with tracer. Cytosol extract binding was normalized to cytosolic protein and nuclear/cytoplasmic binding to DNA. Results are expressed as a percentage of their respective control. Mean ± SEM: $n = 4$ for cytosol extract, and $n = 3$ for whole cells.

Cycloheximide treatment and cellular protein levels

Following 3 h of A23187 treatment (1 μM) there was no significant change in total cellular protein; total protein in control cultures was 100 ± 4 μg and in A23187 treated 94 ± 4 μg (mean ± SEM, $n = 3$). In addition, inhibition of protein synthesis with cycloheximide (30 μM) for 3 h did not mimic the A23187

mediated decrease in [^{3}H]DEX binding; with [^{3}H]DEX binding in control cultures taken as $100 \pm 2\%$, following $30\,\mu M$ cycloheximide treatment binding was $88 \pm 4\%$ (mean $\pm$ SEM: $n = 3$).

BAY K8644 treatment

To further examine the specificity of the calcium induced decrease in GR binding BAY K8644, which promotes extracellular calcium influx via L-type voltage-dependent calcium channels [45], was applied to AtT-20 cells. When cells were treated for 3 h with either 0.1 or $1.0\,\mu M$ of BAY K8644 there was a decrease in [^{3}H]DEX binding to $81 \pm 3\%$ and $76 \pm 3\%$ of control, respectively [Fig. 5(A)], with no concurrent change in DNA recovered [Fig. 5(B)]. In addition, BAY K8644 induced a 3-fold increase in the release of β-endorphin from the same cells [Fig. 5(C)], in agreement with previous studies demonstrating peptide release via L-type calcium channels in AtT-20 cells [46].

PMA treatment

PMA activates PKC and stimulates calcium influx in AtT-20 cells [47]. To assess the possible role activation of PKC has on GR in AtT-20 cells, cells were treated with PMA (100 nM) for various time periods from 0.5 h to 22 h and the level of nuclear localized [^{3}H]DEX binding determined and normalized to DNA recovered. In contrast to the decrease in GR binding demonstrated following an A23187 or BAY K8644 induced calcium influx, there was no rapid change following PMA treatment, although there was a small but significant decrease in binding following 16 h of treatment (Table 2). Immunoreactive β-endorphin levels in the media from the PMA treated cells increased by 40% after 1.5 h of PMA treatment and 47% after 4 h (Table 2), indicating that the cells had been stimulated by PMA.

Table 2. Time course of PMA treatment of AtT-20 cells

	Time (h)	Specific [^{3}H]DEX binding (%)	β-Endorphin (%)
Control	0.5	100 ± 2	NT
PMA		103 ± 1	NT
Control	1.5	100 ± 8	100 ± 4
PMA		92 ± 3	140 ± 15*
Control	4.0	100 ± 3	100 ± 7
PMA		97 ± 3	147 ± 17*
Control	16	100 ± 2	NT
PMA		87 ± 3*	NT
Control	22	100 ± 1	NT
PMA		89 ± 3	NT

AtT-20 cell were treated with 100 nM PMA for the indicated times. Following treatment nuclear [^{3}H]DEX binding was determined and normalized to DNA recovered. In addition, media were collected and assayed for immuno-reactive β-endorphin. Data are expressed as a percentage of control and represent the mean $\pm$ SEM. *$P < 0.05$ compared to respective time control. (NT = not tested).

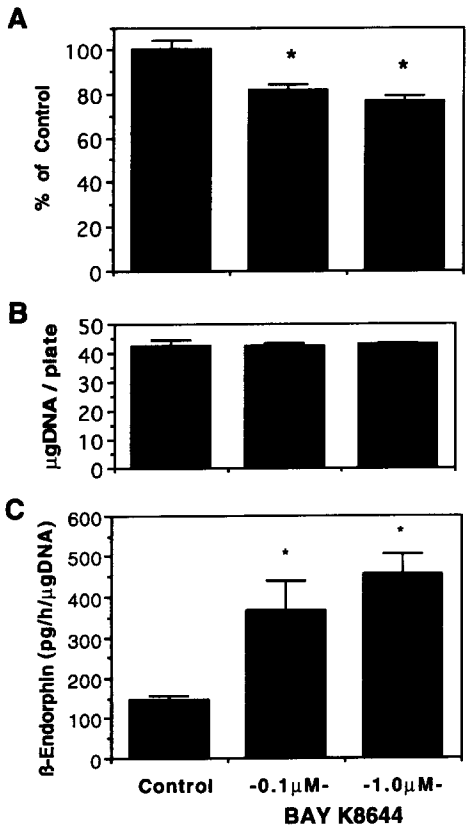

Fig. 5. Effect of BAY K8644 on GR binding (A), DNA recovered (B), and β-endorphin secretion (C) in AtT-20 cells. AtT-20 cells were incubated for 3 h with the indicated concentrations of BAY K8644. Following the incubation, cells were collected and nuclear localized [^{3}H]DEX binding determined, and media collected and analyzed for secreted β-endorphin; both binding and β-endorphin were normalized to DNA recovered. Data are expressed as mean $\pm$ SEM: $n = 4$. *$P < 0.05$.

DISCUSSION

In the present study we have demonstrated that increasing intracellular free calcium either with a calcium ionophore or more specifically via L-type calcium channels can acutely lower the level of GR binding in AtT-20 cells. The observation that the calcium-induced decrease in [^{3}H]DEX binding occurs earlier and is greater than the decrease in GR mRNA would suggest that the initial loss of binding is not due to a decrease in transcription, nor in steady state GR mRNA levels. A calcium induced decrease in the affinity of the GR for DEX has previously been observed in cytosol preparations from rat hepatoma cells [48]. In these studies, addition of micromolar concentrations of $CaCl_2$ to the cytosol preparation decreased GR binding by decreasing the affinity of DEX for the GR [48]. In the light of this observation we compared the affinity of DEX for GR in untreated and A23187 treated AtT-20 cells. Scatchard analysis of [^{3}H]DEX binding in a whole cell preparation following an A23187 induced increase in intracellular calcium demonstrated a decrease in [^{3}H]DEX binding

with no apparent change in the affinity of DEX for the receptor.

Two other possible explanations for the A23187 induced decrease in GR binding is a decrease in receptor number or conversion of the receptor to a non-binding form. A23187 has previously been demonstrated to decrease protein synthesis and overall protein degradation in other cells [49, 50]. However, following 3 h of A23187 treatment AtT-20 total cellular protein did not significantly decrease, and acutely inhibiting protein synthesis with cycloheximide for 3 h did not mimic the A23187 induced decrease in GR binding. Thus, these data indicate that the A23187 induced decrease in GR binding is probably not via the inhibition of GR synthesis, nor via a non-specific decrease in all cellular proteins. The discrepancy in [^{3}H]DEX binding between intact cells and cytosol extract following A23187 treatment is strong evidence that in whole cells A23187 converts the receptor to a non-binding form. The fact that binding can be recovered when cells are lysed and binding measured in cytosol extract indicates that this is not a permanent change in GR, and also that the cellular milieu plays an important role in maintaining the receptor in a non-binding form.

To further characterize the specificity of the calcium effect on GR expression we treated cells with a specific L-type calcium channel agonist BAY K8644. Corticotropes exhibit spontaneous calcium-dependent action potentials [51, 52] which are mediated through two inward calcium currents, a low threshold fast inactivating current (T-type) and a higher threshold slow inactivating current (L-type) [53–55], the latter being sustained open by BAY K8644 [55]. AtT-20 cells treated with BAY K8644 at a concentration (1.0 μM) that induced a three fold increase in β-endorphin secretion, and which has previously been shown to induce a maximal release of β-endorphin from these cells [46], decreased GR binding, though the effect was not as dramatic as that observed for A23187. The difference between A23187 and BAY K8644 treatment on GR expression may be explained by the mixture of L-type calcium channel agonist and antagonist enantiomers of BAY K8644. Even though the antagonistic properties of BAY K8644 on AtT-20 cells are not predominant until concentrations greater than 1.0 μM are used [46], the antagonistic enantiomer may decrease the effectiveness of the agonist at lower concentrations. Another possible explanation is that A23187 increases intracellular calcium through both extracellular influx and release of calcium from intracellular stores [56, 57], whereas BAY K8644 only increases intracellular calcium by extracellular influx and only through L-type calcium channels. Thus the rate of calcium influx and/or the concentration of intracellular free calcium may also be important in the potency of a compound to decrease GR binding.

The GR is a phosphoprotein and the phosphorylation state of the receptor has been implicated in terms of its ability to bind steroid and translocate to the nucleus [58–60]. Activation of PKC has also been proposed to play a role in the phosphorylation state of the GR [61], and recently was demonstrated to down-regulate both estrogen and progesterone receptors in MCF-7 cells [62]. In addition, activation of PKC in corticotropes increases influx of calcium through L-type calcium channels [47, 63]. In contrast to the rapid effects of A23187 and BAY K8644, activation of PKC with the phorbol ester PMA had very little effect on GR binding, though the increase in β-endorphin release observed indicates that PMA did activate PKC in these cells. The inability of PMA to alter GR binding indicates that if phosphorylation of the GR by protein kinase C is occurring, it does not influence the ability of steroid to bind to the receptor. Given that we have measured nuclear localized binding, it is also clear that phosphorylation has not decreased the ability of the steroid/receptor complex to translocate to the nucleus. The fact that PMA has a biphasic effect on calcium influx, in that it initially increases intracellular free calcium (which lasts several minutes) followed by inhibition [47], may explain the inability of a PMA-induced calcium influx to acutely regulate GR binding, and in addition, suggests that a prolonged elevation of calcium may be required. The fact that 16 h of PMA treatment was required to slightly decrease [^{3}H]DEX binding suggests that this decrease is probably not due to the initial transient increase in intracellular free calcium.

We have previously demonstrated that chronic CRF treatment or activation of adenylate cyclase decreases GR binding in AtT-20 cells, and that this effect is mediated, at least in part, through a decrease in steady state mRNA levels [24]. The present study demonstrates that influx of extracellular calcium induces a more rapid and potent decrease in GR binding (A23187 effect reached maximum within 3 h, whereas the CRF effect took 16–24 h). Furthermore, the mechanism involved differs from that induced by CRF, in that there is no initial decrease in GR mRNA levels, and the receptor appears to be present but unable to bind ligand. In addition, the present data have demonstrated that activation of PKC has no apparent effect on GR expression nor the nuclear translocation process in AtT-20 cells. Although the physiological relevance of these results remains to be determined, the association of calcium influx with a rapid decrease in GR binding potentially has very important implications in the ability of GR to regulate many physiological pathways. In terms of the stress response, one can speculate that stress paradigms that lead to chronic release of CRF and/or other factors that induce prolonged elevations of intracellular calcium in corticotropes would lead to desensitization of the negative feedback actions of glucocorticoids at the level of the pituitary. In contrast, those stressors that promote corticotrope ACTH release via activation of PKC (such as AVP) may remain sensitive to glucocorticoid feedback.

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