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Glucocorticoid Receptor Activation Leads to Up-regulation of Adenosine A_1 Receptors and Down-regulation of Adenosine A_2 Responses in DDT_1 MF-2 Smooth Muscle Cells

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SUMMARY

The effect of glucocorticoid treatment of DDT_1 MF-2 smooth muscle cells on the signaling via two adenosine receptors with opposing actions on cAMP generation was examined. Treatment with dexamethasone caused a dose- and time-dependent increase in the number of adenosine A_1 receptors but did not affect the K_D or the proportions of receptors in high and low affinity states. The EC_{50} was 1 nM dexamethasone, and maximal response was achieved after 24 hr. The number of receptors was increased by approximately 50%. Other steroid hormones, including aldosterone, progesterone, testosterone, and estrogen, were much less effective, and addition of the glucocorticoid receptor antagonist RU 486 or the protein synthesis inhibitor cycloheximide prevented the up-regulation, showing that the effect was mediated via a glucocorticoid receptor-specific mechanism that involves protein synthesis. In dexamethasone-treated cells the A_1 receptor agonist (−)-N^6-phenylisopropyladenosine [(*R*)-PIA] was 3 times more potent as an inhibitor of cAMP formation induced by isoprenaline than in untreated cells. ADP ribosylation of inhibitory GTP-binding proteins by pertussis toxin completely prevented (*R*)-PIA from inhibiting cAMP accumulation. A further analysis of the different GTP-binding proteins, including the three G_i subtypes (G_{i1}, G_{i2}, and G_{i3}), revealed no quantitative or qualitative change after dexamethasone treatment. In addition, the adenosine A_2 receptors were down-regulated, as indicated by the fact that the ability of the A_2 receptor agonist 5′-*N*-ethylcarboxamidoadenosine to increase cAMP formation was decreased by 20–30% in dexamethasone-treated cells. In summary, we have shown that A_1 and A_2 receptors on the same cell are differentially regulated by glucocorticoids and that this has functional importance in the regulation of cAMP accumulation.

Glucocorticoids exert a wide variety of physiological effects, including inhibition of inflammatory responses, increase in blood pressure, and modification of mood and behavior, and have wide therapeutic applications (1). Most of these effects are due to interactions with glucocorticoid receptors, which, after translocation to the nucleus, induce specific changes in gene transcription (2). Some of the effects of glucocorticoids are due to interactions with the signaling via membrane receptors that are activated by other agents. For example, treatment with glucocorticoids enhances the signaling induced by activation of β-adrenergic receptors, leading to, among other effects, increased lipolysis in fat cells (3) and increased bronchodilatation (4).

Glucocorticoids have been reported to have the ability to regulate several steps in the signal transduction pathway. The number of receptors is increased in the case of β-adrenoceptors (3–10), insulin receptors (11, 12), and receptors for epidermal growth factor (13). On the other hand, the number of angiotensin II receptors (14) is decreased by dexamethasone treatment. In some systems, increased amounts of stimulatory G proteins (9, 15), as well as increased adenylate cyclase activity (16), have been reported. In addition, cAMP phosphodiesterase activity can be altered (17, 18). Adenosine receptors are of two types, A_1 and A_2, and they have opposing actions on cAMP production (19, 20). Several cells, including cells of the hamster vas deferens smooth muscle cell line DDT_1 MF-2, express both (21, 22). In this particular cell line, increases in the number of β-adrenoceptors after glucocorticoid treatment is well characterized (7, 8, 10). The cell, thus, can alter the expression of surface receptors in response to dexamethasone treatment. Because little is known about the effect of glucocorticoid treatment on

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ABBREVIATIONS: G protein, GTP-binding protein; ADA, adenosine deaminase; BSA, bovine serum albumin; CGS 21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; CHA, N^6-cyclohexyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; G_i, inhibitory GTP-binding protein; G_s, stimulatory GTP-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; NECA, 5′-*N*-ethylcarboxamidoadenosine; PTX, pertussis toxin; (*R*)-PIA, (−)-N^6-phenylisopropyladenosine; RU 486, 17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)estra-4,9-diene-3-one; SDS, sodium dodecyl sulfate; XAC, xanthine amine congener; PBS, phosphate-buffered saline; DTT, dithiothreitol; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

adenosine receptors, we have examined the effect of dexamethasone treatment on the number of adenosine A_1 receptors in DDT_1 MF-2 cells. We have examined whether the change in receptor number leads to a change in the A_1 receptor-mediated inhibition of cAMP accumulation. Further, we examined whether there are any simultaneous changes in the amount of G proteins or in adenylate cyclase activity. Finally, we examined possible simultaneous effects on the adenosine A_2 receptor-mediated cAMP stimulation in DDT_1 MF-2 cells.

Experimental Procedures

Materials. Cell culture media, fetal calf serum, and cell culture flasks were from NordCell (Bromma, Sweden). [^{3}H]DPCPX (80–120 Ci/mmol), [^{32}P]NAD (30 Ci/mmol), [^{3}H]CGS 21680 (48.6 Ci/mmol), [^{3}H]cAMP (44.5 Ci/mmol), and [^{3}H]XAC (128 Ci/mmol) were from New England Nuclear. DPCPX was purchased from Research Biochemicals Incorporated. NECA, CHA, (*R*)-PIA, BSA, cholera toxin, dexamethasone, isoprenaline, and forskolin were all from Sigma. ADA and DTT were purchased from Boehringer (Mannheim, FRG). PTX was from List (Campbell, CA). BW 1433U83UD was a kind gift from Dr. Susan Daluge, Burroughs Wellcome (Research Triangle Park, NC). CGS 21680 was a generous gift from Dr. M. Jarvis, Ciba Geigy (Summit, NJ). RU 486 was a gift from Roussel Nordiska AB (Stockholm, Sweden) via Dr. A. Hansson, Department of Chemistry, Karolinska Institutet (Stockholm, Sweden).

Antiserum against $G_{i\alpha1}$ was a generous gift from Dr. G. Schultz (Institut für Pharmacologie der Freien Universität, Berlin, Germany). Antisera against $G_{i\alpha2}$ and $G_{i\alpha3}$ were from New England Nuclear. Rabbit antiserum raised against a sequence specific for $G_{\alpha s}$ (23) was a generous gift from Dr. A. Spiegel (Department of Health and Human Services, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Rabbit antiserum against a peptide sequence specific for the β subunit [(C)YNLKTREGNVRVSRELAG] (24) was raised in our laboratory.

Cell culture. DDT_1 MF-2 smooth muscle cells, originally isolated from a steroid-induced leiomyosarcoma of Syrian hamster vas deferens (25), were obtained from the American Type Culture Collection. Cells were grown in suspension and maintained in Dulbecco's modified Eagle's medium, with 4.5 g/liter glucose, that contained 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, at 37° in 5% CO_2/95% air. Cells were subcultured (5×10^4 cells/ml) for 12 hr before addition of steroids. Cell viability was >90%, as assessed by the exclusion of trypan blue.

Binding of [^{3}H]DPCPX to intact cells. Cells were harvested by centrifugation for 5 min at 800 × *g*, washed twice in assay medium (serum-free Dulbecco's modified Eagle's medium buffered with 20 mM HEPES, pH 7.4, supplemented with 0.1% BSA), and finally resuspended at a concentration of 1×10^6 cells/ml. Aliquots (0.1 ml) were added to wells in 96-well microtiter plates containing the indicated drugs, in a final volume of 0.3 ml. All drugs were dissolved in the medium described above. Nonspecific binding was defined as that occurring in the presence of 40 μM CHA. Binding was carried out at 25° for 2 hr. Assays were terminated by rapid filtration over filters for receptor binding (Skatron AS, Tranby, Norway). Filtration was performed with a Skatron 1719 cell harvester (Skatron AS), and filters were further washed with 5 ml of ice-cold PBS. Filters were then transferred to scintillation vials (Skatron AS) and, after the addition of 3 ml of scintillation fluid (OptiPhase HiSafe II; LKB, Uppsala, Sweden), counted in a β-counter (1209 Rackbeta; LKB, Uppsala, Sweden).

Binding of [^{3}H]DPCPX to membranes. After cells were washed with PBS, the pellet was resuspended in 1 ml of ice-cold homogenization buffer (50 mM Tris, pH 7.4, 7.5 mM $MgCl_2$, 5 mM EDTA). Cells were disrupted by sonication (MSE 100-W ultrasonic disintegrator; MSE, London) (maximum setting, 4 × 10 sec at 1-min intervals, at 4°). Unbroken cells and nuclei were sedimented at 1000 × *g* for 10 min and discarded. Plasma membranes and the cytosolic fraction were then separated by centrifugation at 30,000 × *g* for 60 min. The membrane pellet was resuspended in assay buffer (50 mM Tris, pH 7.4), at approximately 1 mg of protein/ml, and preincubated with 5 IU/ml ADA for 60 min at 25°, to remove endogenous adenosine. Aliquots (100 μl, 40–50 μg of protein) containing 3 IU/ml ADA were then added to wells in a 96-well microtiter plate, with the indicated drugs, in a final volume of 0.3 ml. Membranes were incubated for 120 min at 25°, and incubations were terminated by filtration over filters for receptor binding, as described for intact cells, with the exception that filters were further washed with assay buffer instead of PBS. Nonspecific binding was defined as that occurring in the presence of 40 μM CHA. Protein determinations were performed with the Bio-Rad protein assay, using BSA as a standard.

cAMP assay. After being washed twice with assay medium, cells were resuspended in the same medium at a density of 1.4×10^5 cells/ml. Aliquots (0.35 ml, 0.05×10^5 cells) were transferred to test tubes, and the indicated drugs were added, together with 30 μM levels of the phosphodiesterase inhibitor rolipram (ZK), to a final volume of 0.5 ml. Reactions were terminated, after 10 min of incubation at 37°, by the addition of perchloric acid to a final concentration of 0.1 M. Samples were neutralized with KOH, and the cAMP content in the supernatants was determined with a protein-binding assay (26), where bound [^{3}H]cAMP was separated from free by rapid filtration over glass fiber filters (Skatron AS).

[^{32}P]ADP-ribosylation of membranes from DDT_1 MF-2 cells with PTX and cholera toxin. Crude membranes from DDT_1 MF-2 cells were prepared by sonication, as described above for receptor binding to membranes. The toxins were preactivated by treatment with 50 mM DTT; PTX was preactivated for 60 min at 25° and cholera toxin for 30 min at 37°. The incubation mixture (100 μl) contained, for PTX treatment, 20 μg/ml PTX, 0.12–2 mg/ml membrane protein, 50 mM Tris·HCl, pH 7.4, 12.5 mM DTT, 1 mM ATP, 10 mM thymidine, 0.5 mM GTP, and 1.1 μM [^{32}P]NAD, and, for cholera toxin treatment, 160 μg/ml cholera toxin, 0.12–2 mg/ml membrane protein, 120 mM potassium phosphate, pH 7.0, 3 mM DTT, 1 mM ATP, 10 mM thymidine, 0.25 mM GTPγS, 10 mM $MgCl_2$, and 1.1 μM [^{32}P]NAD. At the end of the reaction (45 min at 30°), 400 μl of BSA (1 mg/ml) were added, and the reaction was terminated by addition of trichloroacetic acid to a final concentration of 10%. The mixture was kept on ice for 30 min, and the precipitate was washed two times with diethylether (5 min, 13,000 × *g*) and resuspended in Laemmli sample buffer. Samples were boiled and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27), with 10% acrylamide gels. Dried gels were exposed for 2–7 days to Fuji RX film.

Western blotting of G proteins. Crude membrane preparations of DDT_1 MF-2 cells were solubilized in SDS sample buffer (27), and the proteins were separated using SDS-polyacrylamide gel electrophoresis, as described above. After transfer of the proteins with a buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, and 0.1% SDS (pH 8.3), in a semi-dry blotting apparatus (NOVABLOT electrophoretic transfer kit; LKB, Bromma, Sweden), to Immobilon transfer membranes (Millipore, Bedford, MA), the sheets were blocked for 12 hr with 5% (w/v) nonfat dry milk (BLOTTO) in PBS at 4°. The blots were reacted with primary antibody (with the following dilutions: $G_{\alpha s}$, 1:250; $G_{\alpha i1}$, 1:50; $G_{\alpha i2}$, 1:500; $G_{\alpha i3}$, 1:1000) in BLOTTO/PBS for 4 hr at 4°. After three washes with BLOTTO/PBS containing Tween 20 (0.5%), the strips were incubated for 4 hr with anti-rabbit IgG-alkaline phosphatase conjugate (1:1000). After washing, specific bands were developed using 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt) and nitroblue tetrazolium as substrates.

Data analysis. Data from receptor binding studies were analyzed with the commercial version of the LIGAND program (28) adapted for the IBM PC. Dose-response curves were generated by using the GraphPad (ISI Software) program. Statistical comparisons between groups were determined using Student's *t* test or analysis of variance with corrections for multiple comparisons (GraphPad InStat; ISI Soft-

ware). Data are given as mean ± standard error, and each experiment was performed in triplicate.

Results

Effects of dexamethasone treatment on A_1 receptor density and affinity in intact DDT_1 MF-2 cells and in membranes. Dexamethasone increased the B_{max} by approximately 50% (Table 1), as determined from Scatchard plots derived from saturation experiments with [^{3}H]DPCPX. This up-regulation of the receptor number was dose dependent, with the half-maximal effect being observed at 0.8 ± 0.4 nM dexamethasone (four experiments) (Fig. 1). The K_D was unaffected, as determined from Scatchard plots of [^{3}H]DPCPX saturation curves [0.27 ± 0.07 nM in control (eight experiments), compared with 0.29 ± 0.07 nM in dexamethasone-treated cells (seven experiments)] (Fig. 2). To investigate whether dexamethasone

TABLE 1

Effects of steroid hormones, the glucocorticoid receptor antagonist RU 486, and a protein synthesis inhibitor (cycloheximide) on the number of A_1 receptors on intact DDT_1 MF-2 smooth muscle cells

Cells were incubated with the indicated steroid (in combination with antagonist/inhibitor when indicated) for 36 hr. Saturation experiments were then performed with the highly selective adenosine A_1 receptor antagonist [^{3}H]DPCPX. B_{max} values were obtained from corresponding Scatchard plots. Data are presented as mean ± standard deviation, p values compared with control (analysis of variance with corrections for multiple comparisons), and number of experiments (n).

Treatment	B_{max}	p value	n
	fmol/10^6 cells		
Control	119 ± 7		8
Dexamethasone (100 nM)	183 ± 20	<0.0001	7
RU 486 (500 nM)	134 ± 4	>0.05	3
Dexamethasone (100 nM) + RU 486 (500 nM)	137 ± 5	>0.05	3
Cycloheximide (10 μM)	83 ± 5	<0.01	3
Dexamethasone (100 nM) + cycloheximide (10 μM)	88 ± 9	<0.01	4
Aldosterone (500 nM)	134 ± 7	>0.05	3
Progesterone (500 nM)	131 ± 7	>0.05	3
Testosterone (500 nM)	112 ± 9	>0.05	3
Estrogen (500 nM)	117 ± 7	>0.05	3

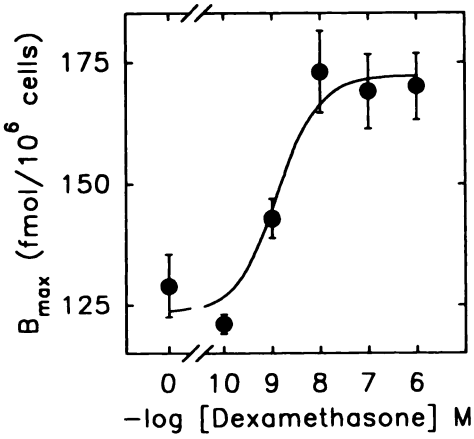

Fig. 1. Dose dependence of dexamethasone-induced increase in adenosine A_1 receptor number in DDT_1 MF-2 smooth muscle cells. Cells were incubated with increasing concentrations of the glucocorticoid dexamethasone for 36 hr. B_{max} was determined from Scatchard plots derived from saturation experiments with [^{3}H]DPCPX as the ligand. Half-maximal effect was achieved with 0.8 ± 0.2 nM dexamethasone. Data presented are means ± standard errors from four experiments, each performed in triplicate.

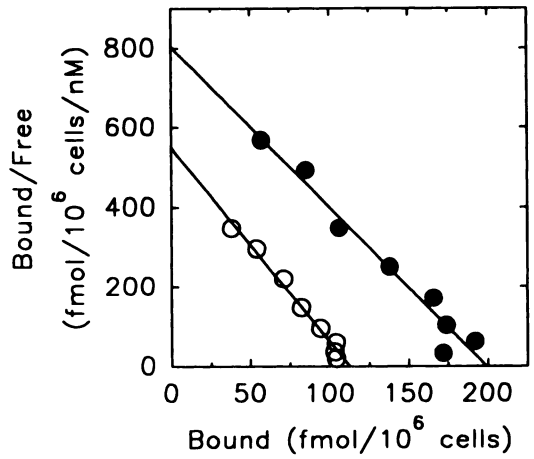

Fig. 2. Effects of dexamethasone on B_{max} and K_D. Treatment of DDT_1 MF-2 smooth muscle cells with 100 nM dexamethasone for 36 hr increased the number of adenosine A_1 binding sites by approximately 50% [119 fmol/10^6 cells in control (eight experiments), compared with 183 fmol/10^6 cells in dexamethasone-treated cells (seven experiments)], without affecting the K_D (0.27 ± 0.07 nM in control, compared with 0.29 ± 0.07 nM in dexamethasone-treated cells). ○, Control cells; ●, dexamethasone-treated cells. B_{max} values were determined from Scatchard plots constructed from saturation experiments with [^{3}H]DPCPX. Data presented are from one representative experiment.

TABLE 2

Influence of dexamethasone treatment on K_D and the relative proportions of receptors in low and high affinity states

Cells were grown in the absence or presence of 100 nM dexamethasone for 36 hr. Binding was then performed to either membranes (six experiments) or intact cells (three experiments), where (*R*)-PIA competed with [^{3}H]DPCPX (1 nM) for binding.

Treatment	Affinity		Receptor proportion	
	K_H	K_L	High affinity	Low affinity
	nM		%	
Membranes				
None	1.7 ± 1.4	90 ± 32	55 ± 6	45 ± 6
Dexamethasone (100 nM, 36 hr)	1.2 ± 0.6	97 ± 42	54 ± 9	46 ± 6
Intact cells				
None		128 ± 25		100
Dexamethasone (100 nM, 36 hr)		113 ± 18		100

treatment caused any change in the relative proportions of receptors in the high and low affinity forms, we prepared membranes and performed competition studies in which (*R*)-PIA competed with [^{3}H]DPCPX for binding. These studies revealed no change in high or low affinity K_D values or in the relative proportions of receptors in high and low affinity states (Table 2). The response to 100 nM dexamethasone was maximal after 24 hr of treatment but could already be detected after 8 hr (Fig. 3). It was possible to displace all bound [^{3}H]DPCPX (1 nM) with the highly hydrophilic adenosine A_1 antagonist BW 1433U83UD (data not shown), showing that the increase in B_{max} was due to an increase in cell surface receptors. In order to rule out the possibility that treatment with glucocorticoids changed the amount of endogenous adenosine, we added a high concentration (5 IU/ml) of ADA. This did not change the B_{max} or the K_D (data not shown). Addition of the glucocorticoid receptor antagonist RU 486 or the protein synthesis inhibitor cycloheximide abolished the up-regulation by dexamethasone (Table 1). Cycloheximide itself caused a reduction in B_{max}

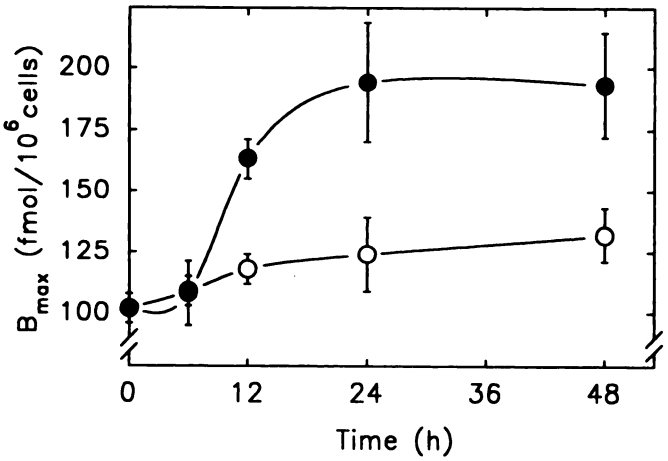

Fig. 3. Time course for the up-regulation of adenosine A_1 receptors in DDT_1 MF-2 smooth muscle cells after treatment with dexamethasone. B_{max} values were determined from [^{3}H]DPCPX saturation experiments in control cells (○) and cells treated with 100 nM dexamethasone (●), at the indicated time points. Data are given as means ± standard errors from four experiments, each performed in triplicate.

TABLE 3

cAMP accumulation after direct activation of G_s proteins and adenylate cyclase

Control cells and cells treated with 100 nM dexamethasone for 36 hr were activated with cholera toxin (5 μg/ml, 2 hr) and forskolin (10 μM, 10 min). There was no significant difference between control cells and dexamethasone-treated cells (95% confidence interval, *t* test). Data are from three independent experiments, each performed in triplicate (mean ± standard error).

Treatment	cAMP	
	Control	Dexamethasone-treated
	pmol/10⁶cells	
Control (10 min)	48 ± 6	44 ± 2
Forskolin (10 μM, 10 min)	432 ± 47	522 ± 65
Cholera toxin (5 μg/ml, 120 min)	1037 ± 27	1013 ± 129

(Table 1). Addition of other steroid hormones (aldosterone, progesterone, testosterone, and estrogen) at concentrations 500 times their K_D values did not cause any significant change in B_{max} (Table 1).

Ability of (*R*)-PIA to inhibit cAMP formation after dexamethasone treatment. Basal levels of cAMP were not changed by 36 hr of treatment of cells with 100 nM dexamethasone (Table 3). β-Adrenergic stimulation of cAMP accumulation by 10 μM isoprenaline was greater after dexamethasone treatment (527 ± 39 pmol/10^6 cells in control experiments, compared with 666 ± 33 pmol/10^6 cells in treated cells; three experiments). To investigate whether endogenous adenosine could accumulate to sufficient levels to inhibit isoprenaline-induced cAMP accumulation, we added ADA (5 IU/ml), preincubated the cells for 30 min at 37°, and performed the experiment in the presence of 1.5 IU/ml ADA. ADA treatment did not change basal or isoprenaline-induced cAMP accumulation (data not shown). The same result (i.e., no interference) was observed when cells were treated with PTX (200 ng/ml, 4 hr) and when experiments were performed in the presence of 1 μM DPCPX.

Direct activation of G_s proteins with cholera toxin (5 μg/ml, 2 hr) or of the adenylate cyclase itself with forskolin (10 μM, 10 min) did not produce any significant differences between treated and untreated cells (Table 3).

The potency of the A_1 receptor agonist (*R*)-PIA as an inhibitor of cAMP formation induced by isoprenaline (10 μM) was increased 3-fold, with a shift in the IC_{50} from 8.4 ± 1.7 nM in control experiments to 2.7 ± 0.59 nM in cells treated with 100 nM dexamethasone for 36 hr ($p < 0.05$, three experiments) (Fig. 4). The maximal effect of (*R*)-PIA was not altered. Treatment of cells with PTX completely abolished the ability of (*R*)-PIA to inhibit cAMP formation (Fig. 4).

Adenosine A_2 receptor function after dexamethasone treatment. The A_2 receptor agonist NECA was unable to stimulate cAMP accumulation in both control cells and dexamethasone-treated cells (100 nM, 36 hr) (Fig. 5). NECA is able to activate A_1 as well as A_2 receptors, and there are 4 times as many A_1 as A_2 receptors in DDT_1 MF-2 cells (22). We previously (21) showed that this inability was due to a strong inhibition of A_2 receptor-mediated cAMP accumulation, due to the simultaneous activation of the A_1 receptor, which could be prevented by pretreatment of cells with PTX (200 ng/ml, 4 hr). In the present study, therefore, we used such ADP-ribosylation of inhibitory G proteins to reveal functional A_2 receptors (Fig. 5). Dexamethasone treatment decreased the NECA-induced formation of cAMP by approximately 20% ($p < 0.05$) (Fig. 5). The same effect (reduction by 25–30%, $p < 0.05$) was also seen when A_1 receptors were blocked by the highly selective A_1 receptor antagonist DPCPX (1 μM) (Fig. 6). The adenosine A_2-selective agonist CGS 21680 did not elevate cAMP levels in DDT_1 MF-2 cells. Inclusion of 1 μM DPCPX in the experiments, to rule out a possible interaction with A_1 receptors, did not produce any cAMP accumulation in response to CGS 21680.

Analysis of G proteins. In order to investigate whether the observed changes in the responses mediated via A_1 and A_2

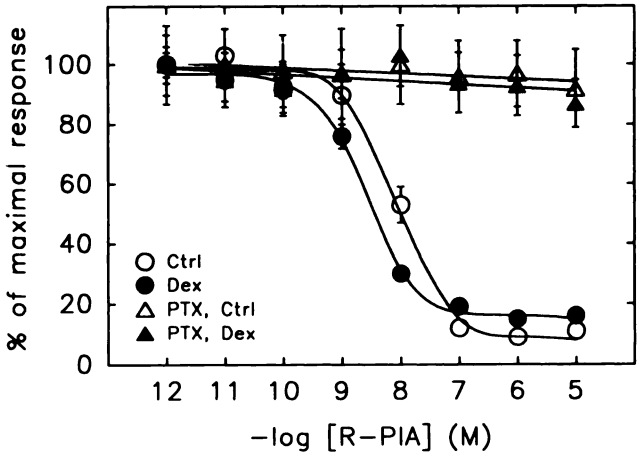

Fig. 4. (*R*)-PIA-mediated inhibition of cAMP formation in intact DDT_1 MF-2 smooth muscle cells. The figure shows the ability of (*R*)-PIA to inhibit the formation of cAMP induced by 10 μM levels of the β-adrenergic agonist isoprenaline in control cells (○) and dexamethasone-treated cells (100 nM, 36 hr) (●). Basal levels were unchanged by dexamethasone treatment (59 ± 7 pmol/10^6 cells in control, compared with 61 ± 7 pmol/10^6 cells in dexamethasone-treated cultures). The response to 10 μM isoprenaline was increased after treatment (527 ± 39 pmol/10^6 cells in control experiments, compared with 666 ± 33 pmol/10^6 cells in dexamethasone-treated cells). Results are expressed as percentage of inhibition of isoprenaline-stimulated cAMP accumulation. (*R*)-PIA was 3 times more potent in inhibiting isoprenaline (10 μM)-induced cAMP accumulation, with a leftward shift in IC_{50} from 8.4 ± 1.7 nM to 2.7 ± 0.59 nM (*t* test, 95% confidence interval). Treatment with PTX (200 ng/ml, 4 hr) completely prevented (*R*)-PIA from inhibiting cAMP accumulation (△, control; ▲, dexamethasone). Data are means ± standard errors for three experiments performed in triplicate, assayed in duplicate.

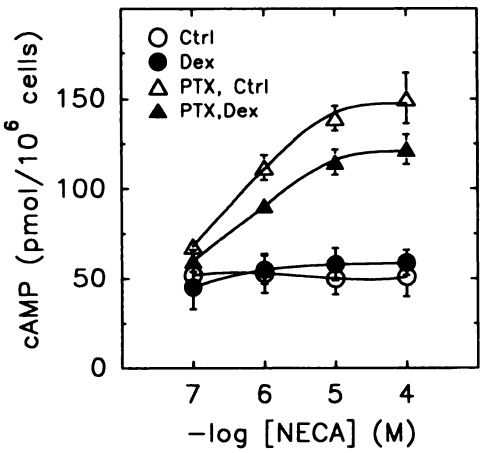

Fig. 5. Influence of dexamethasone treatment on adenosine A_2 responses in intact DDT_1 MF-2 smooth muscle cells. The ability of increasing concentrations of the A_2 receptor agonist NECA to stimulate cAMP accumulation in control (○) and dexamethasone-treated (100 nM, 36 hr) cells (●) is shown. Without pretreatment with PTX, NECA was unable to increase cAMP levels. However, after pretreatment of cells with 200 ng/ml PTX for 4 hr (△, control; ▲, dexamethasone), NECA was able to stimulate cAMP formation. In dexamethasone-treated cells, there was a 20% reduction in the maximal accumulation of cAMP ($p < 0.05$). Results are from five experiments, each performed in triplicate; samples were assayed in duplicate. Points represents means ± standard errors.

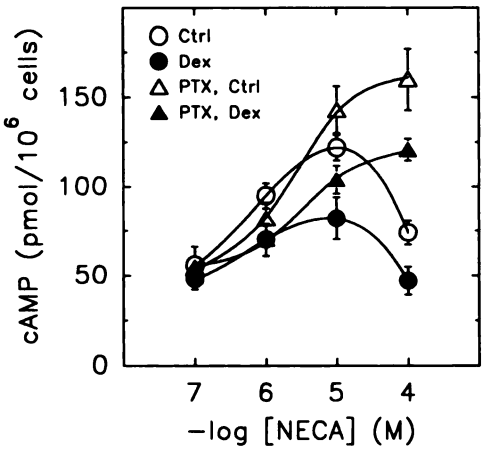

Fig. 6. NECA-induced cAMP formation in the presence of DPCPX. NECA-induced cAMP accumulation in the presence of 1 μM levels of the A_1 receptor antagonist DPCPX in control cells (○, △) and dexamethasone-treated cells (100 nM, 36 hr) (●, ▲) is shown. The ability of NECA to induce cAMP formation was evaluated in both PTX-treated cells (△, ▲) and untreated cells (○, ●). Dexamethasone treatment resulted in a 25–30% reduction in cAMP response ($p < 0.05$). At higher concentrations, NECA was able to overcome the effect of 1 μM DPCPX. Data are means ± standard errors from three or four experiments performed in triplicate, assayed in duplicate.

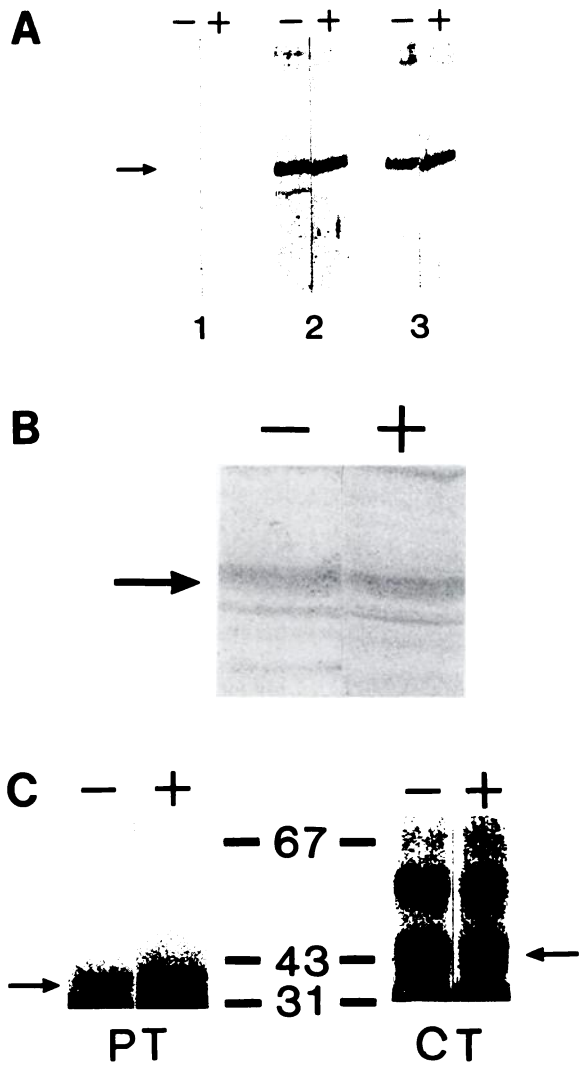

Fig. 7. Quantitation of G proteins in membranes from DDT_1 MF-2 smooth muscle cells, using immunoblotting and ADP-ribosylation. Membranes were prepared from control cells (−) and cells treated with 100 nM dexamethasone for 36 hr (+). The amounts of α subunits from G_i and G_s proteins were visualized as described below. Densitometric analysis with subtraction of background revealed no quantitative difference between treated and nontreated membranes (data not shown) in any α subunit investigated. A, Immunoblot of $G_{i\alpha}$ subunits. G_i protein subtypes were visualized with antisera against αi1, αi2, and αi3 subunits, as described in Experimental Procedures. Each lane was loaded with 150 μg of membrane protein. *Arrow*, 41–42 kDa. B, Visualization of the α_s subunit with immunoblotting. Each lane was loaded with 62 μg of membrane protein. *Arrow*, 45 kDa. C, ADP ribosylation of G proteins with PTX and cholera toxin. Membranes were incubated with [^{32}P]NAD in the presence of cholera toxin (*CT*) or PTX (*PT*) and were then subjected to SDS-gel electrophoresis, as described in Experimental Procedures. Each lane was loaded with 100 μg of membrane protein.

receptors could be due to changes in the amount of G proteins, we used immunoblotting with several peptide antibodies against α subunits and ADP-ribosylation with cholera toxin or PTX. Inhibitory G proteins were investigated with antibodies against three α subunits (αi1, αi2, and αi3). DDT_1 MF-2 cells did not contain detectable levels of $G_{\alpha i1}$, and there was no clear cut quantitative or qualitative change in $G_{\alpha i2}$ or $G_{\alpha i3}$ after dexamethasone treatment (Fig. 7A). Nor did we find any change in $G_{\alpha s}$ (Fig. 7B) or in the amount of the β subunit (data not shown). ADP-ribosylation with PTX or cholera toxin also did not reveal any quantitative change in G_i or G_s after dexamethasone treatment (Fig. 7C).

Discussion

We have shown that dexamethasone increases the number of adenosine A_1 receptors and increases the effect of A_1 receptor stimulation on cAMP formation. Although there are many reports of glucocorticoid receptor-induced changes in receptors that stimulate cAMP formation, this is one of the first examples of an up-regulation of a receptor that inhibits cAMP. There

was a 50% increase in the receptor number, which was paralleled by a 3-fold increase in the potency of the A_1 agonist (*R*)-PIA to inhibit adenylate cyclase. There was no apparent change in the amount of G protein subunits. Thus, the A_1 receptor system in DDT_1 MF-2 cells shows the characteristics of a system with spare receptors (29). It is important to point out that, in one of the few other instances where the consequences of adaptive up-regulation of a G_i-coupled receptor system have been examined, namely, in muscarinic receptors in the central nervous system, an increase in receptor number was associated with a decreased responsiveness of the receptor-coupled second messenger system (30, 31). It has been reported that treatment of cells with dexamethasone can alter the relative proportions of receptors in low and high affinity states (4, 32), which, in turn, could be responsible for functional changes in the coupling to adenylate cyclase. When we investigated this possibility, we found no change in the relative proportions of receptors of low and high affinity or in K_D values.

The potency of dexamethasone to up-regulate A_1 receptors agrees with its binding to DDT_1 MF-2 smooth muscle cells (33) and to brain glucocorticoid receptors (34) and with its potency to cause up-regulation of β-adrenergic (3), dopamine (16), and prostaglandin E_1 responses (35, 36). The up-regulation of adenosine A_1 receptors on DDT_1 MF-2 smooth muscle cells was blocked by the glucocorticoid receptor antagonist RU 486 (37) and was poorly or not at all mimicked by other steroid hormones.

The time course was similar to that observed earlier for other systems that depend on glucocorticoid receptor activation [β-adrenergic (5), DA (16), prostaglandin E_1 (35, 38), glucagon (39), and epidermal growth factor receptor (13)]. Even though the magnitude of the up-regulation of adenosine A_1 receptors was somewhat smaller than that commonly observed for β-adrenergic-receptors (e.g., Refs. 5–10), it is likely that the underlying mechanism is similar.

It has been demonstrated that the increase in receptor density is preceded by an increase in the amount of mRNA encoding for the receptor. This has been shown for β-adrenergic (8, 10) and insulin receptors (11). Addition of protein synthesis inhibitors blocked this increase in β-adrenergic receptors and mRNA (10), but for the insulin receptor only the receptor synthesis was blocked, without any effect on mRNA increase after glucocorticoid treatment (11). This suggests that glucocorticoids are able to regulate specific genes that encode for the receptors and the translation of the receptor from the corresponding mRNA.

In the present experiments, the protein synthesis inhibitor cycloheximide prevented the up-regulation by dexamethasone. Even though data concerning cycloheximide should be interpreted with caution, because the synthesis of many other proteins, including the glucocorticoid receptor itself, could be blocked by cycloheximide (40), the data indicate that the transcription or translation of the A_1 receptor could be regulated by glucocorticoids. Cycloheximide decreased the number of A_1 receptors. A reduction in receptor-mediated responses by cycloheximide has been seen in other systems (9, 16, 35, 36, 38, 39) and could reflect receptor turnover, as has been shown for the β-adrenergic receptor (5).

In other systems, it has been shown that glucocorticoids can increase the amount of G proteins (9, 15), which could explain an enhanced hormone-stimulated cAMP accumulation. Increased levels of cAMP via direct stimulation of G proteins with GTP (3, 7, 9, 15, 32, 38), cholera toxin (9, 16, 32, 35), or NaFl (9, 38) have been shown after treatment with glucocorticoids. These data have also been interpreted as increased levels of the G_s protein or increased affinity for GTP. We were not able to detect any quantitative or qualitative change in the G_i ($G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{\alpha i3}$) or the G_s protein in DDT_1 MF-2 cells using either immunoblotting or cholera toxin/PTX-mediated ADP-ribosylation techniques. Although there is one report of increased amounts of the G_i protein in osteosarcoma cells (9), this author was not able to show that there was any receptor coupled to this protein in this cell line, and glucocorticoid regulation of receptors coupled to an inhibitory G protein has not been reported.

Whereas glucocorticoid treatment increased the number of A_1 receptors and, thereby, the signaling via the A_1 receptor, it decreased by 20–30% the ability of the adenosine A_2 receptor agonist NECA to stimulate cAMP formation. This is unlikely to be due to a change in G_s (see above) or adenylate cyclase or phosphodiesterase activities (17, 18), because β-adrenoceptor responses are enhanced. Furthermore, the present experiments were carried out in the presence of a phosphodiesterase inhibitor, and neither cholera toxin nor forskolin responses were affected by dexamethasone treatment. Therefore, the response is most likely due to an effect on the adenosine A_2 receptor. We have not been able to confirm the proposed effect on A_2 receptors with binding techniques, because we could not detect any binding of commercially available labeled ligands (CGS 21680 and XAC) to membranes or intact cells, which would indicate that the A_2 receptors on DDT_1 MF-2 cells are of the A_{2b} subtype.

In conclusion, the present study has demonstrated a glucocorticoid receptor-mediated increase in the number of G_i-coupled A_1 receptors in DDT_1 MF-2 cells that leads to an increased potency of A_1 receptor agonists as inhibitors of cAMP accumulation. At the same time, the efficacy of A_2 receptors to activate cAMP accumulation is decreased, possibly due to a decreased expression of A_2 receptors. Thus, in DDT_1 MF-2 smooth muscle cells, glucocorticoid treatment will shift the balance between two receptors to the same autocoid that have opposing actions on cAMP formation.

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