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Sensitization by dexamethasone of lymphocyte cyclic AMP formation: evidence for increased function of the adenylyl cyclase catalyst

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1 Glucocorticoids and elevations of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) may affect lymphocyte activation, proliferation and effector functions in similar ways. Therefore, we have investigated the effects of the glucorticoid, dexamethasone, on human lymphocyte cyclic AMP formation.

2 Treatment of resting human lymphocytes with the glucocorticoid, dexamethasone, sensitized prostaglandin E_2-stimulated cyclic AMP accumulation in a time- and concentration-dependent manner.

3 In membranes of lymphocytes treated for 24 h with 100 nM dexamethasone, maximal adenylyl cyclase activity stimulated by prostaglandin E_2, isoprenaline, guanosine 5'-triphosphate (GTP), forskolin and $MnCl_2$ was significantly enhanced; the EC_{50} for these agents was not significantly altered.

4 β_2-Adrenoceptor density, immunodetectable α-subunits of the G-proteins G_s and G_i, and pertussis toxin-substrates were not significantly altered by dexamethasone treatment.

5 In dexamethasone-treated lymphocytes, prostaglandin E_2-mediated inhibition of concanavalin A-induced Ca^{2+} elevations was doubled compared to control cells.

6 Based on these data and the observation that enhancement of forskolin- and $MnCl_2$-stimulated adenylyl cyclase activity could quantitatively account for the enhancement of prostaglandin E_2-, isoprenaline- or GTP-stimulated adenylyl cyclase activity, we conclude that dexamethasone treatment sensitizes cyclic AMP formation in resting human lymphocytes by altering the adenylyl cyclase catalyst rather than G-proteins or hormone receptors. This results in an enhanced capability of cyclic AMP generating agonists to inhibit early steps of lymphocyte activation.

Keywords: Glucocorticoid; lymphocyte; adenylyl cyclase; G-protein; immunomodulation

Introduction

Glucocorticoids are potent immunosuppressive drugs the mechanism of action of which appears to involve inhibition of early steps in the activation of resting lymphocytes (Flower, 1988). An increase in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) can also suppress lymphocyte proliferation and effector functions by inhibiting early steps of their activation (Bourne *et al.*, 1974; Kammer, 1988). For example both cyclic AMP elevation and glucocorticoids inhibit expression of interleukin-2 (Northrop *et al.*, 1992; Anastassiou *et al.*, 1992) and interleukin-2 receptors (Tracey *et al.*, 1988; Anastassiou *et al.*, 1992) at a transcriptional level. In non-lymphoid tissues, glucocorticoids can enhance cyclic AMP formation (Malbon *et al.*, 1988). Formation of cyclic AMP results from the complex interaction of receptors, G-proteins and the adenylyl cyclase catalyst (Levitzki, 1988), and expression and/or functional activity of each component of this complex might be affected by glucocorticoids. For example, the gene for the human β_2-adrenoceptor which is expressed in all types of lymphocytes to stimulate cyclic AMP formation (Brodde *et al.*, 1987; Maisel *et al.*, 1989) contains a glucocorticoid response element (Hadcock *et al.*, 1989). Expression of the α-subunit of the adenylyl cyclase-stimulating G-protein G_S can also be enhanced by glucocorticoids in various non-lymphoid cell lines (Rodan & Rodan, 1986; Rizzoli & Bonjour, 1987; Chang & Bourne, 1987). Whether glucocorticoids can also affect expression of the adenylyl cyclase catalyst is not known.

Based on these observations we and others have hypothesized that cyclic AMP may at least partly be involved in the immunosuppressant effects of glucocorticoids (Michel & Brodde, 1989; Gruol *et al.*, 1989). The present study has investigated (a) whether the synthetic glucocorticoid dexamethasone can enhance cyclic AMP formation in human cultured resting lymphocytes, (b) which part(s) of the receptor/G-protein/adenylyl cyclase complex might be regulated by dexamethasone, and (c) whether enhanced cyclic AMP formation following glucocorticoid treatment is relevant for the modulation of lymphocyte function.

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Methods

Lymphocyte isolation and cell culture

Mononuclear leukocytes were isolated from EDTA-anticoagulated venous blood of drug-free healthy young volunteers of either sex according to the method of Böyum (1968). Unless otherwise indicated cells were incubated for 24 h in an atmosphere of 95% air/5% CO_2 in RPMI 1640 medium supplemented with 2 mM glutamine, 25 μg ml^{-1} gentamycin and 20% newborn bovine serum in the absence and presence of the indicated dexamethasone concentrations. Following this incubation, non-adherent mononuclear leukocytes (lymphocytes) were washed twice at 400 *g* for 10 min in phosphate-buffered saline.

Cyclic AMP accumulation in intact lymphocytes

Cyclic AMP accumulation in intact lymphocytes was determined during a 15 min incubation at 37°C in the presence of 100 μM of the phosphodiesterase inhibitor, theophyllin as previously described (Brodde *et al.*, 1985). The accumulated cyclic AMP was quantified with a commercially available protein binding assay (Amersham, Braunschweig, Germany).

Adenylyl cyclase activity in lymphocyte membranes

Lymphocyte membranes were prepared by three cycles of freeze/thawing using liquid nitrogen and a water bath at 37°C with subsequent centrifugation for 10 min at 29,000 *g* at 4°C as previously described (Maisel *et al.*, 1990b). The pellet was resuspended by repeated aspiration through a cannula (inner diameter 0.8 mm) yielding membranes of ≈250,000 cells per 20 µl sample. The protein content of the membrane preparation was assessed according to Bradford (1976) using bovine IgG as the standard.

Adenylyl cyclase activity was determined as previously described (Michel *et al.*, 1993b). Briefly, aliquots of the membrane suspension (≈10–14 µg protein) were incubated for 10 min at 37°C in a total volume of 100 µl containing 25 mM Tris, 5 mM $MgCl_2$, 0.5 mM adenosine 5'-triphosphate (ATP), 10 µM guanosine 5'-triphosphate (GTP), 20 µg creatine phosphate and 25 u ml^{-1} creatine phosphokinase at pH 7.4 and the indicated concentrations of agonists. The incubation was stopped by boiling, with subsequent cooling of the samples and centrifugation for 10 min at 13,000 *g*. Following storage at – 20°C the amount of cyclic AMP in the supernatant was determined by a commercially available radioimmunoassay (New England Nuclear, Dreieich, Germany).

Lymphocyte β-adrenoceptor density

β_2-Adrenoceptor density in intact lymphocytes was determined as previously described using six concentrations of [^{125}I]-iodocyanopindolol as the ligand (Brodde *et al.*, 1985). Non-specific binding was defined as binding in the presence of 1 µM of the hydrophilic antagonist (±)-CGP 12,177 and typically was 20% of total binidng of 20 pM of the ligand.

Lymphocyte G-proteins

Lymphocyte G-protein α-subunits were quantified by Western blotting and pertussis toxin-catalyzed ADP-ribosylation. Western blotting was performed according to the method of Burnette (1981) with minor modifications as previously described (Michel *et al.*, 1993a,b). Briefly, membranes were prepared from lymphocytes that had been incubated in the absence and presence of dexamethasone as described above and resuspended at a protein concentration of 2 mg ml^{-1}. Aliquots of the membrane suspension (50 µl) were boiled for 5 min together with 12.5 µl aliquots of sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mM Tris HCl, 0.002% bromphenol blue at pH 8). The mixture was separated by electrophoresis on polyacrylamide/SDS gels according to the method of Laemmli (1970) using 10% acrylamide in the running gel. On gels for the determination of $G_{s\alpha}$ we loaded one lane with membranes from cyc$^-$ cells which lack $G_{s\alpha}$ (Sternweis & Gilman, 1979) in order to control for non-specific bands. Following electroblotting to nitrocellulose membranes (Hybond-ECL 0.45 µm, Amersham), the blots were pre-blocked for 90 min at room temperature with TBS buffer (100 mM NaCl, 20 mM Tris HCl at pH 7.5) supplemented with 2% low-fat dried milk powder. Following two 5 min washes in TTBS (TBS supplemented with 500 µl l^{-1} Tween-20) at room temperature, blots were incubated for 20 h at 4°C in 15 ml TTBS supplemented with 1% dried milk powder and a 1:500 dilution of G-protein antisera (AS/7 for $G_{i\alpha}$ and RM/1 for $G_{s\alpha}$). Following removal of the antisera suspension the blots were washed twice for 10 min each at room temperature in TTBS. Thereafter, each blot was incubated with 100 ml TTBS supplemented with 1% dried milk powder and 80 µl ^{125}I-labelled protein A solution (8.5 µCi $µg^{-1}$, 129 µCi ml^{-1}) for 1 h at room temperature. Following four 10 min washes with TTBS, blots were dried, wrapped in plastic foil and used for autoradiography. Specific bands were identified on the autoradiograms, and corresponding bands were cut from the blots and the incorporated radioactivity was determined in a scintillation counter. Radioactivity incorporated into bands from lanes where no membranes had been loaded was defined as non-specific binding and was subtracted from the total binding.

Pertussis toxin-catalyzed ADP-ribosylation was carried out as previously described in detail (Maisel *et al.*, 1990b). Briefly, aliquots of the lymphocyte membrane suspension (10 µl at 1 mg protein ml^{-1}) were incubated with aliquots of pre-activated pertussis toxin (13 µl at 18 µg toxin ml^{-1}) and reaction mixture (13 µl containing as final concentrations: 139 mM TrisHCl at pH 8, 13.9 mM thymidine, 1.4 mM ATP, 0.14 mM GTP, 3.4 mM $MgCl_2$, 1.4 mM EDTA, 13.9 mM dithiothreitol, 692 µM NADP and 1.4 µM [^{32}P]-NAD) for 1 h at 30°C. The reaction was stopped by addition of 36 µl sample buffer with subsequent boiling. Electrophoretic separation of proteins was performed on gels containing 17% acrylamide. Incorporation of radioactivity into bands from lanes where membranes and reaction mixture had been loaded without pertussis toxin was taken as non-specific incorporation and was subtracted from that in all other lanes.

Ca^{2+} measurements

Intracellular Ca^{2+} concentrations were determined using the fluorescent indicator dye Fura-2 with excitation alternating between 340 and 380 nm and emission recorded at 510 nm in a Hitachi F-2000 spectrofluorometer; experiments were performed in Krebs-Henseleit buffer containing 108 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, and 11 mM glucose. Data were analysed by the ratio method with software provided by the fluorometer manufacturer; details have been described previously (Michel *et al.*, 1992).

Chemicals

[^{32}P]-NAD, [^{125}I]-protein A and the antisera RM/1 and AS/7 were from New England Nuclear, Fura-2 from Molecular Probes (Eugene, OR, U.S.A.), RPMI 1640 and L-glutamine were from ICN (Eschwege, Germany), foetal and newborn bovine serum were from Boehringer Mannheim (Mannheim, Germany), (±)-CGP 12,177 (1-[2-(3-carbamoyl-4-hydroxy) phenoxyethylamino]-3- [4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulphonate) was a gift of Ciba Geigy (Basel, Switzerland), and all other chemicals were from Sigma (Munich, Germany).

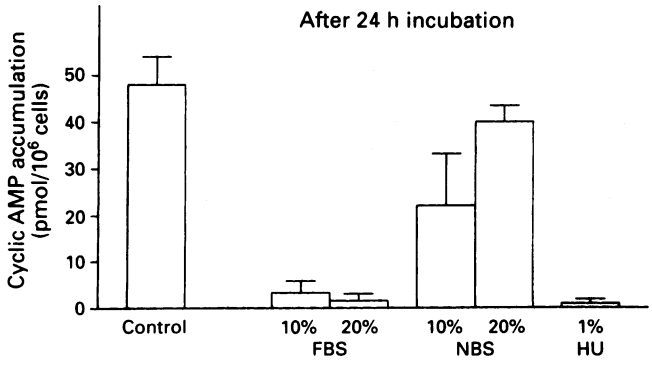

Figure 1 Effects of media supplements on prostaglandin E_2-stimulated cyclic AMP accumulation: Cyclic AMP accumulation stimulated by 10 µM prostaglandin E_2 was determined in intact freshly prepared lymphocytes (control) and in lymphocytes cultured for 24 h in RPMI medium supplemented with the indicated concentrations of foetal or newborn bovine serum (FBS and NBS, respectively) or the chemically defined medium supplement nutridoma HU. Data are mean ± s.e.mean of three experiments.

Data evaluation

Data are presented as mean ± s.e.mean. Statistical significance of differences was assessed by Student's paired t test if two groups were compared and by one-way analysis of variance followed by t tests with Bonferroni correction for multiple comparisons if more than two groups were compared; all statistical analysis was performed using the InStat programme (GraphPAD Software, San Diego, CA, U.S.A.) and a $P<0.05$ was considered significant. Concentration-response curves were analysed by non-linear regression analysis with interative fitting of the experimental data to sigmoidal curves using the InPlot programme (GraphPAD Software); in order to optimize estimates for EC_{50} and maximal effects a constant Hill-slope of one was assumed in these calculations. Pooled data of all experiments were fitted simultaneously, and the statistical significance of differences in EC_{50} and maximal effects were assessed by comparing the 95% confidence intervals (CI) for these parameters obtained during the fitting procedure. Additionally, the concentration-response curves of control and treated cells were compared by two-way analysis of variance to test whether treatment had significantly affected cyclic AMP formation at the $P<0.05$ level.

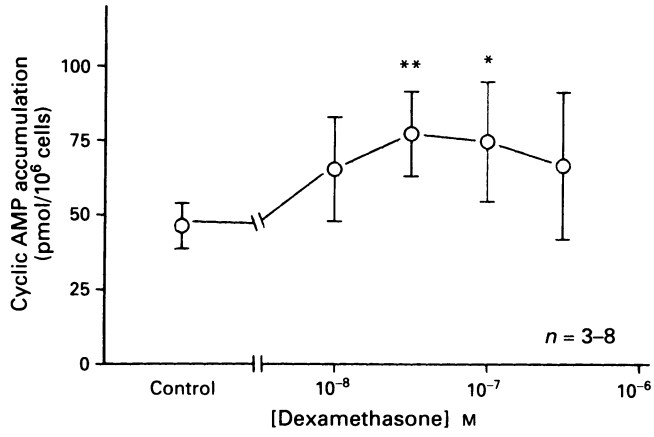

Figure 2 Effect of dexamethasone on prostaglandin E_2-stimulated cyclic AMP accumulation: Lymphocytes were cultured for 24 h in the absence (Control) or presence of the indicated concentrations of dexamethasone. Following washing of the cells, cyclic AMP accumulation stimulated by 10 μM prostaglandin E_2 was determined in intact lymphocytes. Data are mean ± s.e.mean of 3–8 experiments. $*P<0.05$, $**P<0.01$ vs. control in a paired two-tailed t test.

Results

In the first part of our study, we determined optimal conditions to assess glucocorticoid modulation of lymphocyte cyclic AMP formation by measuring 10 μM prostaglandin E_2-stimulated cyclic AMP accumulation in intact lymphocytes. Previous studies have demonstrated that resting human lymphocytes in culture rapidly lose their ability to generate cyclic AMP upon hormonal stimulation (Maisel *et al.*, 1989). Whereas 10% newborn and 10% and 20% foetal bovine serum or the chemically-defined serum substitute nutridoma HU, failed to prevent loss of prostaglandin-stimulated cyclic AMP formation, addition of 20% newborn bovine serum almost completely prevented it (Figure 1). Thus, all further lymphocyte incubations were performed in the presence of 20% newborn bovine serum. Under these conditions prostaglandin-stimulated cyclic AMP accumulation typically ranged between 40–50 pmol per 10^6 cells 15 min^{-1}.

We then tested whether and under what conditions the synthetic glucocorticoid, dexamethasone, enhanced prostaglandin-stimulated cyclic AMP accumulation in intact human lymphocytes. Incubation with 100 nM dexamethasone for 24 h enhanced prostaglandin-stimulated cyclic AMP accumulation by 24.0 ± 6.8 pmol per 10^6 cells 15 min^{-1} ($n=12$, $P=0.0049$). Shorter incubations resulted in smaller and statistically not significant enhancements (data not shown). Thus, a 24 h incubation was chosen for all further experiments. In concentration-response experiments maximal enhancement of prostaglandin-stimulated cyclic AMP accumulation occurred between 30 and 100 nM of dexamethasone (Figure 2). Thus, 100 nM of dexamethasone was used in all further experiments. This enhancing effect was specific for glucocorticoids since other steroid hormones including oestradiol, 5α-dihydro-testosterone and progesterone did not enhance prostaglandin-stimulated cyclic AMP accumulation (data not shown).

To define which part(s) of the receptor/G-protein/adenylyl cyclase catalyst complex had been regulated by dexamethasone, adenylyl cyclase stimulation experiments were performed in membranes obtained from dexamethaxone- and vehicle-treated lymphocytes. Dexamethasone treatment enhanced maximal prostaglandin E_2-stimulated adenylyl cyclase activity in lymphocyte membranes from 290 (95% CI: 221–359) to 561 (95% CI: 490–632) pmol mg^{-1} protein 10 min^{-1} ($P<0.0001$ in a two-way analysis of variance; Figure 3). The EC_{50} of prostaglandin E_2 was 24.3 μM (− log EC_{50} 4.61, 95% CI: 4.97–4.26) in control and 9.1 μM (− log EC_{50} 5.04, 95% CI:

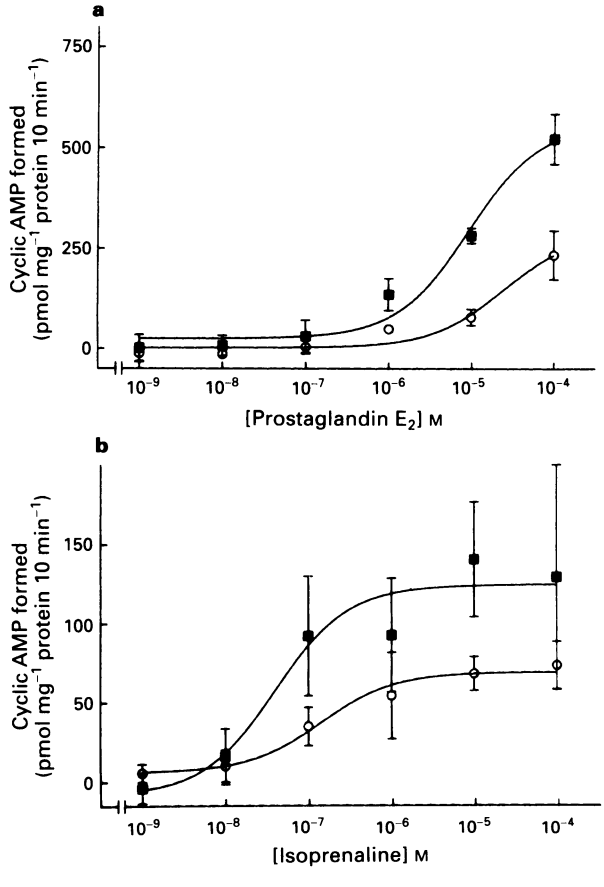

Figure 3 Effects of dexamethasone treatment on hormone-stimulated adenylyl cyclase activity: lymphocytes were cultured for 24 h in the absence (○) and presence (■) of 100 nM dexamethasone. Adenylyl cyclase activity was stimulated in membranes from these cells by prostaglandin E_2 (a) or isoprenaline (b). Basal adenylyl cyclase activity in the presence of 10 μM GTP has been subtracted from each value. Data are mean ± s.e.mean of four experiments. Concentration-response curves for prostaglandin E_2- and isoprenaline-stimulated cyclic AMP formation are significantly enhanced in membranes from dexamethasone-treated cells with $P<0.0001$ and $P=0.0180$, respectively, as determined by two-way analysis of variance.

5.28–4.80) in treated membranes (Figure 3). Similarly isoprenaline-stimulated adenylyl cyclase activity was enhanced from 68 (95% CI: 64–73) to 124 (95% CI: 109–138) pmol mg^{-1} protein 10 min^{-1} ($P = 0.0180$ in a two-way analysis of variance; Figure 3). The EC_{50} for isoprenaline was 143 nM (– log EC_{50} 6.84, 95% CI: 7.06–6.63) in control and 40 nM (– log EC_{50} 7.40, CI: 7.83–6.97) in treated membranes (Figure 3).

Adenylyl cyclase stimulation by the receptor-independent G-protein activator, GTP (10 μM) was enhanced by glucocorticoid treatment from 236 ± 19 to 309 ± 27 pmol mg^{-1} protein 10 min^{-1} ($n = 29$, $P < 0.05$). Dexamethasone treatment also significantly enhanced maximal adenylyl cyclase stimulation by forskolin from 620 (95% CI: 606–635) to 949 (95% CI: 879–1020) pmol mg^{-1} protein 10 min^{-1} ($P = 0.0063$ in a two-way analysis of variance; Figure 4). The potency (– log EC_{50}) of forskolin was not significantly altered (6.18 [95% CI: 6.25–6.11] in membranes from control and 6.07 [95% CI: 6.27–5.87] in those from treated cells; Figure 4). Dexamethasone-treatment also significantly enhanced maximal adenylyl cyclase stimulation by $MnCl_2$ from 371 (95% CI: 347–394) in control to 673 (95% CI: 645–701) pmol mg^{-1} protein 10 min^{-1} in treated cells ($P = 0.0074$ in a two-way analysis of variance; Figure 4). Dexamethasone treatment did not significantly alter the EC_{50} of $MnCl_2$ which was 5.2 mM (– log EC_{50} 2.28, 95% CI: 2.41–2.15) in control and 6.9 mM (– log EC_{50} 2.16, 95% CI: 2.23–2.08) in treated cells (Figure 4). Thus dexamethasone enhancement of adenylyl cyclase stimulation at the level of the catalyst was quantitatively similar to those at the G-protein or receptor level, indicating that the glucocorticoid had mainly regulated the adenylyl cyclase catalyst.

Therefore, we tested directly whether glucocorticoid regulation of adenylyl cyclase-coupled receptors and/or G-proteins were also detectable. Lymphocyte β_2-adrenoceptor density was determined under three conditions: in freshly prepared cells and in those cultured 24 h in the presence of vehicle or dexamethasone. Lymphocytes from each volunteer were always tested in parallel under all three conditions. Neither β_2-adrenoceptor density (Figure 5) nor affinity for the radioligand differed significantly among groups (15.9 ± 0.5 pM in fresh cells, 17.0 ± 1.8 pM in vehicle-treated cells, and 15.4 ± 0.7 pM in dexamethasone-treated cells, $n = 4$). Thus, glucocorticoid regulation of lymphocyte β_2-adrenoceptors was not detectable under our conditions.

Next we assessed the effect of dexamethasone treatment on lymphocyte G-protein α-subunit expression. The $G_{s\alpha}$ antiserum RM/1 recognized a double band in lymphocyte membranes (apparent molecular weights 42 and 44 kDa) with the lower band dominating. Both bands were specific since they were not seen in membranes from cyc^- cells (data not shown). The $G_{i\alpha}$ antiserum AS/7 recognized a single band with an apparent molecular weight of 39 kDa. Pertussis toxin catalyzed the incorporation of ^{32}P into a single band with an apparent molecular weight of 39 kDa. The amounts of ^{125}I-labelled protein A and ^{32}P incorporated into these specific bands were similar in membranes from control and dexamethasone-treated cells in all three assays (Table 1). Thus, glucocorticoid regulation of lymphocyte α-subunits of G_s or G_i was not detectable under our conditions.

Finally, we tested the relevance of sensitized cyclic AMP formation for hormonal modulation of lymphocyte function. An early event in prostaglandin E_2 suppression of lymphocyte activation is inhibition of mitogen-stimulated Ca^{2+} increases which is mediated by cyclic AMP (van Tits *et al.*, 1991; Michel *et al.*, 1992). The inhibition of concanavalin A-stimulated Ca^{2+} increases by 1 μM prostaglandin E_2 was doubled in dexamethasone-treated compared to vehicle-treated lymphocytes (Figure 6).

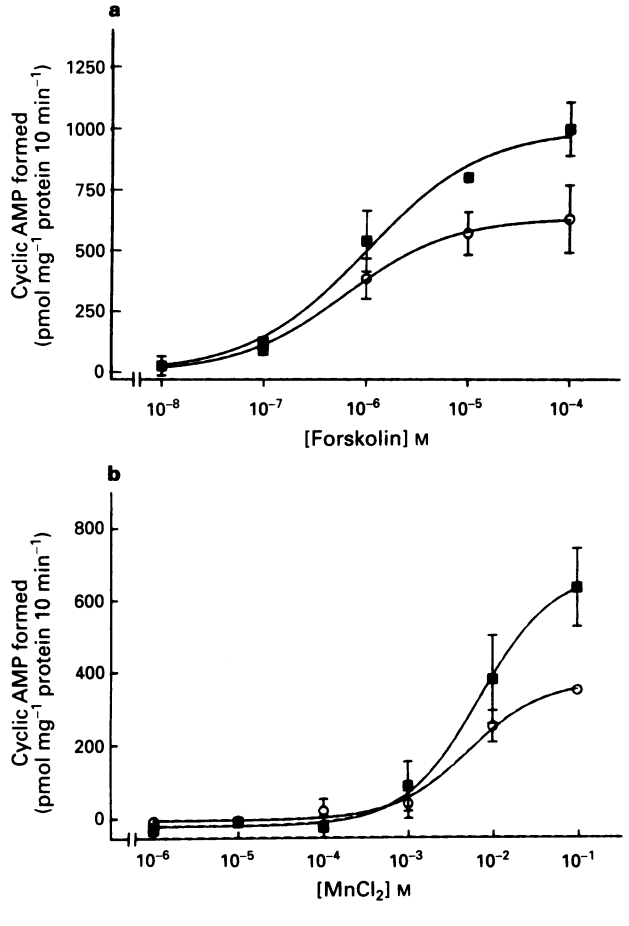

Figure 4 Effects of dexamethasone treatment on receptor-independent stimulation of adenylyl cyclase: lymphocytes were cultured for 24 h in the absence (○) and presence (■) of 100 nM dexamethasone. Adenylyl cyclase activity was stimulated in membranes from these cells by forskolin (a) or $MnCl_2$ (b). Basal adenylyl cyclase activity in the presence of 10 μM GTP has been subtracted from each value. Data are mean ± s.e.mean of four experiments. Concentration-response curves for forskolin- and $MnCl_2$-stimulated cyclic AMP formation are significantly enhanced in membranes from dexamethasone-treated cells with $P = 0.0063$ and $P = 0.0074$, respectively, as determined by two-way analysis of variance.

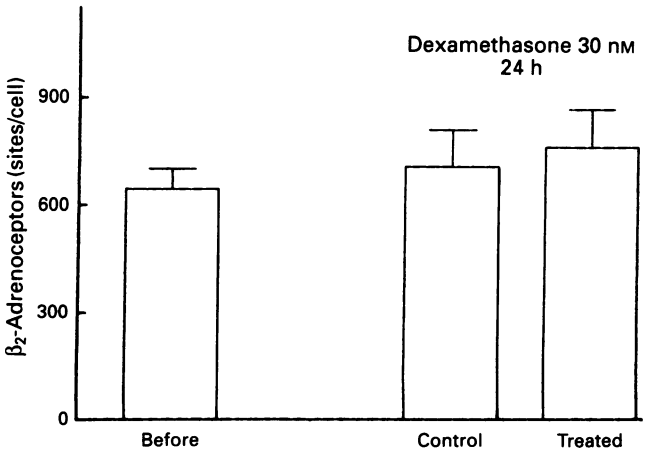

Figure 5 Effect of dexamethasone treatment on lymphocyte β_2-adrenoceptor density: lymphocyte β_2-adrenoceptor density was determined by saturation binding experiments with [^{125}I]-iodocyanopindolol in freshly prepared lymphocytes (Before), and cells which had been cultured for 24 h in the absence (Control) or presence (Treated) of 30 nM dexamethasone. Data are mean ± s.e.mean of four paired experiments and are expressed as [^{125}I]-iodocyanopindolol binding sites/cell.

Table 1 Effect of dexamethasone treatment on lymphocyte G-protein α-subunits

	Control	*Dexamethasone-treated*
Immunodetectable $G_{s\alpha}$	27,277 ± 2490	31,165 ± 2097
Immunodetectable $G_{i\alpha}$	97,937 ± 5741	100,425 ± 8750
Pertussis toxin substrates	4,608 ± 645	5,300 ± 885

Resting human lymphocytes were cultured for 24 h in the absence (control) or presence of 100 nM dexamethasone. Thereafter, G-protein α-subunits were determined in a membrane preparation by quantitative Western blotting or pertussis toxin-catalyzed ADP-ribosylation. Data are mean ± s.e.mean of six experiments. Immunodetectable α-subunits are given as c.p.m. of [^{125}I]-protein A specifically bound per mg lymphocyte membrane protein, pertussis toxin substrates are given as fmol ^{32}P specifically incorporated per mg membrane protein. None of the differences was statistically significant.

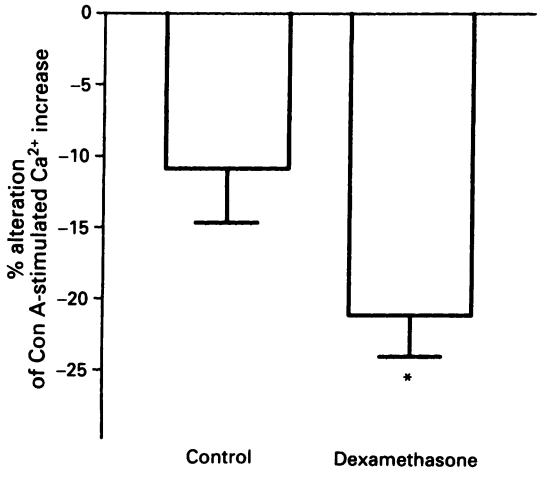

Figure 6 Effects of dexamethasone treatment on inhibition of concanavalin A-stimulated Ca^{2+} increases by prostaglandin E_2: 30 s following addition of 1 μM prostaglandin E_2 or vehicle, concanavalin A (100 μg ml^{-1})-stimulated Ca^{2+} increases were determined in Fura-2 loaded lymphocytes. We calculated % inhibition by the prostaglandin from Ca^{2+} increases in its absence and presence. Parallel experiments were performed in cells which had been cultured for 24 h in the absence (Control) or presence of 100 nM dexamethasone. Data are mean ± s.e.mean from 12 paired experiments with a duplicate determination each in the absence and presence of the prostaglandin. *$P<0.05$ in dexamethasone- vs. vehicle-treated lymphocytes.

Discussion

The cyclic AMP/cyclic AMP-dependent protein kinase system is an important modulator of lymphocyte activation, proliferation, differentiation and effector functions (Bourne *et al.*, 1974; Kammer, 1988). Formation of cyclic AMP is controlled by a cascade consisting of receptors, the stimulatory and inhibitory G-proteins G_s and G_i, and the adenylyl cyclase catalyst. Expression of each component can be dynamically regulated to alter the overall responsiveness of the cascade. Glucocorticoids exert a permissive effect on cyclic AMP-elevating agents in various cell types (Malbon *et al.*, 1988). Therefore, we hypothesized that glucocorticoids might exert their immunomodulatory effects at least partly by affecting cyclic AMP formation.

Previous work had demonstrated that resting human lymphocytes in culture rapidly lose their ability to generate cyclic AMP in response to hormonal stimulation (Maisel *et al.*, 1989). Our data demonstrate that in contrast to a number of other medium supplements, addition of 20% newborn bovine serum can at least partly prevent that loss of responsiveness. Although this addition was sufficient to detect and quantify prostaglandin E_2-stimulated cyclic AMP generation, these conditions may still be suboptimal compared to freshly isolated cells. This may limit the extrapolation of our data to *in vivo* conditions.

Using these culture conditions we demonstrate that the synthetic glucocorticoid, dexamethasone enhances prostaglandin E_2-stimulated cyclic AMP formation in human cultured lymphocytes. Similar enhancement has previously been detected in non-lymphoid cell types (Rodan & Rodan, 1986; Rizzoli & Bonjour, 1987; Chang & Bourne, 1987). The time- and concentration-dependency for the enhancing effect of dexamethasone in human lymphocytes were similar to those reported for dexamethasone effects in other model systems, notably for the enhancement of hormone-stimulated cyclic AMP formation in ROS 17/2.8 cells (Rodan & Rodan, 1986). Optimal stimulatory conditions were then used to determine which parts of the receptor/G-protein/adenylyl cyclase cascade are affected by dexamethasone exposure. This was done in two ways: on the one hand, we performed functional tests on adenylyl cyclase responsiveness using agonists which act on different levels of the receptor/G-protein/adenylyl cyclase cascade; on the other hand, we quantified directly individual components such as receptors and G-protein subunits.

We have used the receptor agonists, prostaglandin E_2 and isoprenaline, to test the whole cascade, GTP as a direct G-protein activator, forskolin as a direct adenylyl cyclase catalyst activator which also has some G-protein effects, and Mn^{2+} as a direct adenylyl cyclase catalyst activator. Treatment with dexamethasone did not significantly alter the potency of any agonist. On the other hand dexamethasone enhanced the maximal adenylyl cyclase responses to all tested agonists. These enhancements were quantitatively similar for all agonists. Since forskolin and $MnCl_2$ stimulate adenylyl cyclase distal to receptors and G-proteins, our data indicate that dexamethasone treatment might primarily affect the adenylyl cyclase catalyst. Whether this is due to increased expression of the catalyst, to an altered enzymatic activity of the enzyme, or both cannot be decided from the present data.

To support this indirect conclusion, we have also directly tested whether dexamethasone treatment affects the expression of lymphocyte β_2-adrenoceptors and/or G-protein α-subunits. Our data demonstrate that dexamethasone-treatment did not significantly alter the number of β_2-adrenoceptors (which mediate the stimulatory effects of isoprenaline) in our model. Glucocorticoids also failed to alter human lymphocyte β_2-adrenoceptor expression *in vivo* (Brodde *et al.*, 1985). The observation that human lymphocyte β_2-adrenoceptors are not regulated by glucocorticoids despite the presence of a glucocorticoid-response element in the regulatory domain of the human β_2-adrenoceptor gene (Hadcock *et al.*, 1989) may be related to the known tissue-dependency of glucocorticoid effects (Malbon *et al.*, 1988).

Cyclic AMP formation could be sensitized at the G-protein level by increased G_s or decreased G_i. Confirming the above functional data, direct quantification of G_s and G_i α-subunits by immunoblotting and ADP-ribosylation did not detect significant increases in G_s or decreases in G_i expression. In RBL-2H3 cells, a rat cell line which is used as a model for mast cells, dexamethasone-treatment also failed to alter expression of G_s or G_i at the protein or the mRNA level (Hide *et al.*, 1991). In contrast glucocorticoids enhance G_s expression in three cell lines which are not related to the white blood cell lineage, ROS 17/2.8 osteosarcoma cells (Rodan & Rodan, 1986), OK-cells derived from opossum kidney (Rizzoli & Bonjour, 1987) and GH_3-cells derived from rat pituitary (Chang & Bourne, 1987). We cannot explain the different behaviour of these cell types at present but speculate that it may be related to the fact that lymphocytes and RBL-2H3 cells belong to stem cell lineage whereas the other cell types do not. The question remains why glucocorticoids

enhance adenylyl cyclase activity in human lymphocytes at the level of the catalyst but apparently do not alter catalyst activity in other model systems. Although we cannot conclusively answer this question at present, we speculate that it may be related to the tissue-specificity of expression of adenylyl cyclase isoforms (Tang & Gilman, 1992). Whether and how such isoforms are differentially regulated by glucocorticoids remains to be studied.

In the final part of our study we asked whether enhanced hormonal stimulation of cyclic AMP formation in lymphocytes following dexamethasone treatment is functionally relevant. We have previously shown that prostaglandins and other cyclic AMP elevating agents inhibit mitogen-induced Ca^{2+} elevations in human lymphocytes (van Tits *et al.*, 1991; Michel *et al.*, 1992). This effect is indeed mediated by cyclic AMP since it is enhanced in the presence of phosphodiesterase inhibitors (van Tits *et al.*, 1991). Our present data demonstrate that the ability of a submaximally effective prostaglandin E_2 concentration to inhibit concanavalin A-stimulated Ca^{2+} increases is almost doubled in dexamethasone-treated lymphocytes. Similarly, cyclic AMP elevation by prostaglandin E_2 or forskolin and the glucocorticoid, methylprednisolone, enhance each others effects on DNA fragmentation and viability in mouse thymocytes (McConkey *et al.*, 1993). Finally, the glucocorticoid cytotoxicity against T-lymphocytes depends on the amount of cyclic AMP-dependent protein kinase present in such cells (Gruol *et al.*, 1989).

Taken together our data demonstrate that glucocorticoids enhance lymphocyte cyclic AMP formation by affecting the catalyst of adenylyl cyclase and that this enhancement is functionally relevant *in vitro*. Elevations of lymphocyte cyclic AMP inhibit lymphocyte activation, proliferation and effector functions *in vitro* (Bourne *et al.*, 1974; Kammer, 1988). *In vivo* cyclic AMP elevating agents such as the β-adrenoceptor agonists cause lymphopaenia in man (Maisel *et al.*, 1990a) and reduce splenic weight in rats (Murray *et al.*, 1993), whereas β-adrenoceptor agonists can increase the number of circulating T-cells in man (Maisel *et al.*, 1991). Whether enhanced lymphocyte cyclic AMP formation is at least partly relevant for the immunosuppressive effects of glucocorticoids *in vivo* cannot be answered from the present *in vitro* data. However, prostaglandin E_1-, GTP-, and forskolin-stimulated adenylyl cyclase activity are also enhanced in lymphocytes obtained from patients treated with glucocorticoids *in vivo* (Michel & Brodde, 1989). Thus, the mechanisms discussed above may also operate *in vivo* in a clinical setting. Their functional role for immunosuppression remain to be assessed.

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