Increase of adenylate cyclase catalytic-unit activity by dexamethasone in rat osteoblast-like cells

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Glucocorticoids are known to increase the cyclic AMP response to parathyroid hormone (PTH) in cultured bone organs or bone cells. Using the osteoblast-like cell line ROS 17/2.8, which possesses receptors for both PTH and glucocorticoids, we investigated which component of the complex hormone receptor–guanine nucleotide regulatory unit–adenylate cyclase was affected by dexamethasone treatment. In response to PTH, isoproterenol or forskolin, a compound that is supposed to act directly on the catalytic unit, cyclic AMP production by intact cells and adenylate cyclase activity in purified plasma membrane were markedly increased by dexamethasone. Whereas NaF, guanosine 5'-[$\beta\gamma$ -imido]triphosphate and Mn^{2+} stimulated adenylate cyclase activity were similarly enhanced in membranes isolated from glucocorticoid-treated cells, the activity of the stimulatory guanine nucleotide regulatory unit, as assessed by reconstitution into membranes from the CYC⁻ clone, which is genetically devoid of this component, was not altered. Thus in osteoblast-like cells dexamethasone appears to increase cyclic AMP synthesis by influencing the catalytic unit. Moreover, since it has been reported that glucocorticoids may produce changes in cell calcium metabolism, we evaluated cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and intracellular Ca^{2+} stores mobilizable by the bivalent-cationophore ionomycin, by using the intracellular fluorescent indicator Quin-2. The results indicated that dexamethasone treatment did not influence $[Ca^{2+}]_i$ but markedly decreased ionomycin-releasable Ca^{2+} stores.

INTRODUCTION

It is generally recognized that cellular effects of steroid hormones are not mediated by the cyclic AMP system, as opposed to those of several peptide hormones. However, there is evidence that steroid hormones can modulate the cyclic AMP response to various agonists (Foster & Perkins, 1977; Davies & Lefkowitz, 1984). Moreover, in cultured bone explants, as well as in cultured bone cells, glucocorticoids increase the PTH-induced stimulation of cyclic AMP production (Chen & Feldman, 1978, 1979, 1984; Hahn & Halstead, 1979; Ng *et al.*, 1979; Rodan *et al.*, 1984; Catherwood, 1985). Thus, since cyclic AMP generation may be associated with decreased osteoblastic function and higher bone resorption (Dietrich *et al.*, 1976), this could contribute to osteopenia observed *in vivo* under glucocorticoid treatment.

The hormone-sensitive adenylate cyclase system is a multi-component membrane-associated complex, consisting of the hormone receptor, a catalytic unit which synthesizes cyclic AMP, and a guanine nucleotide regulatory protein (G-unit) which links the two previous components (Gilman, 1984; Schramm & Selinger, 1984). Hence a glucocorticoid-induced enhancement of the cyclic AMP response to PTH could result from a modification of any of these three components. By using specific stimulators for each compound of the adenylate

cyclase system and by direct measurements of the guanine nucleotide regulatory protein using the reconstitution assay, experiments were carried out to localize the site which may have become affected by the glucocorticoid. The studies were performed with the osteoblast-like rat osteosarcoma cell line ROS 17/2.8, which was shown to possess receptors for both PTH and glucocorticoids (Majeska *et al.*, 1980; Haussler *et al.*, 1980; Rizzoli *et al.*, 1983).

Previously it has been reported that cells treated with glucocorticoids change their Ca^{2+} metabolism (Rasmussen & Gustin, 1978; Hällgren *et al.*, 1985). Since the cyclic AMP response to PTH appeared to be enhanced by Ca^{2+} in bone cells (Peck *et al.*, 1981), we investigated whether the stimulatory effect of dexamethasone on the PTH-induced cyclic AMP production could be accompanied by alteration of the intracellular Ca^{2+} metabolism.

MATERIALS AND METHODS

Materials

Native bovine PTH-(1–84)-peptide (2200 units/mg) was kindly provided by Dr. T. M. Murray (Division of Endocrinology and Metabolism, St. Michael's Hospital, Toronto, Canada). Dexamethasone was a gift from Lepetit, Milano, Italy. Forskolin was obtained from Calbiochem–Behring Corp. p[NH]ppG was purchased from Boehringer-Mannheim. [α - ^{32}P]ATP and cyclic

Abbreviations used: p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; PTH, parathyroid hormone (parathyrin); G-unit, guanine nucleotide regulatory unit; IBMX, 3-isobutyl-1-methylxanthine; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration.

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[³H]AMP was from New England Nuclear. Dulbecco's modified Eagle's minimal essential medium, Ham's F₁₂ and Dulbecco's phosphate-buffered saline were purchased from Seromed, and fetal-calf serum was obtained from Boehringer Mannheim or North America Biologicals. Quin 2/AM was purchased from Amersham.

Cell culture and membrane preparation

The clonal rat osteosarcoma-cell lines ROS 17/2.8 and ROS 24/1 were kindly provided by Dr. G. Rodan and Dr. R. Majeska (University of Connecticut Health Center, School of Dental Medicine, Farmington, CT, U.S.A.). The cells were seeded at a density of approx. 1.3×10^4 cells/cm², in a 1:1 mixture of Ham's F₁₂/Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum containing 100 units of penicillin/ml and 100 µg of streptomycin/ml. Medium was replaced on days 3, 6, 8 and 9. Incubations with dexamethasone or ethanol (0.1%) were carried out during the last 2 days in culture. To avoid a major influence on cell growth and the possible predominance of a cell subpopulation, the influence of dexamethasone was studied only with the cells at confluence in carefully controlled and similar conditions with respect to time in culture and seeding density. When intact cells were used, they were removed from the flask with 0.02% EDTA and 0.05% trypsin on day 10 or 11 (24 h after the last replacement of medium). Soya-bean trypsin inhibitor (Sigma) was immediately added, and subsequently the cells were washed twice at 4 °C with the incubation medium described below. For plasma-membrane isolation, cell layer was washed twice with 10 mM-Tris/HCl/1 mM-EDTA/0.25 M-sucrose, pH 7.4, and scraped into the same buffer. Cells were homogenized by 15 strokes with a Teflon pestle. After centrifugation at 750 g for 5 min, the supernatant was centrifuged at 11000 g for 20 min and pellet obtained, containing the plasma membranes, was stored in liquid N₂. Before use, the membranes were washed by resuspension in ice-cold 10 mM-Tris/HCl/1 mM-EDTA, pH 7.4.

Cells from the CYC⁻ clone of S49 mouse lymphoma (kindly provided by Dr. R. W. Downs, Jr., National Institutes of Health, Bethesda, MD, U.S.A.), which are genetically deficient in G-unit activity (Bourne *et al.*, 1975), were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) horse serum. Cells were harvested by centrifugation and resulting pellet was washed twice in Dulbecco's phosphate-buffered saline. Plasma membranes were prepared by incubating the cells for 30 min at 4 °C in a hypo-osmotic buffer containing 2 mM-Tris/HCl/1 mM-EDTA/1 mM-dithiothreitol, pH 7.4. Homogenization and isolation of plasma membranes were carried out as described above. Membranes were stored in liquid N₂ at a concentration of 3–8 mg/ml.

Cyclic AMP production and adenylate cyclase assay

For the determination of cyclic AMP production, incubations were carried out at 37 °C for 7 min in a volume of 250 µl of Dulbecco's phosphate-buffered saline containing 20 mM-Hepes, pH 7.4, 0.9 mM-CaCl₂, 1 mM-MgCl₂, 0.1% heat-inactivated bovine serum albumin, 0.1% glucose, 0.2 mM-IBMX and 1×10^5 – 2×10^5 cells (Rizzoli *et al.*, 1983). Incubations were terminated by the addition of 250 µl of cold 10% (w/v) HClO₄. The suspension was subsequently neutralized with 1 M-

KHCO₃, pH 8.3. After centrifugation, total cyclic AMP was determined as described previously (Brown *et al.*, 1971). Serial dilutions of the samples produced curves which paralleled that of the standard curve.

Adenylate cyclase activity in osteosarcoma-cell membranes was assayed for 10 min at 37 °C in a final volume of 100 µl containing 30 mM-Tris/HCl (pH 7.4), 2 mM-MgCl₂ unless otherwise indicated, 1 mM-dithiothreitol, 0.5 mM-cyclic AMP, 0.1 mM-ATP, 200000–400000 c.p.m. of [α -³²P]ATP, 0.1% heat-inactivated albumin, 1.8 mg of phosphocreatine/ml, 0.2 mg of creatine kinase/ml and 10–40 µg of membrane protein. Incubations were terminated by adding 0.1 ml of an ice-cold solution containing 30 mM-Tris/HCl, pH 7.4, 0.5 mM-cyclic AMP, 40 mM-ATP and 4000–8000 c.p.m. of cyclic [³H]AMP. The mixture was boiled for 3 min, and cyclic AMP was isolated by the procedure of Salomon *et al.* (1974).

Measurement of G-unit activity by adenylate cyclase complementation assay

Osteoblastic-cell membranes isolated from ethanol-treated control or steroid-treated cells were pelleted by centrifugation (4000 g for 20 min) in 10 mM-Tris/HCl/1 mM-EDTA, pH 7.4, and solubilized during an overnight incubation at 4 °C in 10 mM-Tris/HCl, pH 7.4, containing 0.25 M-sucrose, 0.1 mM-MgCl₂, 1 mM-dithiothreitol and 0.2% Lubrol PX. The membrane suspension was then centrifuged at 47000 g for 60 min. The supernatant (membrane extract) was collected and assayed for G-unit activity. The pellets containing control and steroid-treated cell membranes were also solubilized during an overnight incubation at 4 °C in 30 mM-Tris-HCl, pH 8.0, containing 0.25 M-sucrose, 0.1 mM-MgCl₂, 1 mM-dithiothreitol and 1% cholate. After centrifugation for 60 min at 47000 g, supernatant cholate extract was collected and diluted 10-fold in buffer without cholate before assay of G-unit activity.

To measure G-unit activity, Lubrol PX or cholate extracts was added to CYC⁻ membranes, and the reconstituted p[NH]ppG- or NaF-stimulated adenylate cyclase activity was determined (Downs *et al.*, 1983). This was carried out by incubating 5, 10, 15 and 20 µl of membrane detergent extract for 20 min at 30 °C with 60–90 µg of CYC⁻ membranes in a volume of 90 µl containing 30 mM-Tris/HCl, pH 7.4, 5 mM-MgCl₂, 1 mM-dithiothreitol, 0.5 mM-cyclic AMP, 0.1 mM-ATP, 1.8 mg of phosphocreatine/ml and 0.2 mg of creatine kinase/ml with 10 mM-NaF or 100 µM-p[NH]ppG. The detergent concentration in all cases was kept constant by adding an appropriate volume of solubilizing buffer. At the end of the incubation period, 10 µl containing 0.2–0.3 µCi of [α -³²P]ATP was added for an additional 20 min incubation at 30 °C. Cyclic [³²P] AMP formed was isolated and determined (Salomon *et al.*, 1974). Membrane extracts alone did not exhibit any measurable adenylate cyclase activity in response to the agonists. The assay of G-unit activity was linear with respect to the amount of extract protein added.

Measurement of cytoplasmic free calcium ([Ca²⁺]_i)

Confluent osteoblast-like rat osteosarcoma cells were removed from culture dishes with trypsin/EDTA as described above after 48 h exposure to either 100 nM-dexamethasone or 0.1% ethanol. Then they were incubated (10^6 cells/3 ml) in Dulbecco's modified Eagle's

medium containing 2% fetal-calf serum under constant stirring for 2–3 h at 37 °C. The cells were washed twice in 20 mM-Hepes/140 mM-NaCl/1 mM- Na_2HPO_4 containing 1 mM- CaCl_2 , 1 mM- MgSO_4 , 5 mM-KCl, 5.5 mM-glucose, pH 7.4, and subsequently resuspended at a concentration of 5×10^6 cells/ml in this buffer supplemented with 0.2% albumin at 37 °C. The cells were loaded with the fluorescent indicator Quin-2 by adding 100 μM of the membrane-permeant compound Quin-2 acetoxy-methyl ester from a 10 mM stock solution in anhydrous dimethyl sulphoxide. After 20 min, the indicator was diluted by adding 3 vol. of albumin-free buffer and the incubation continued for 40 min. The cells were then washed twice by centrifugation and resuspension. Fluorescence of Quin-2-loaded cells (10^6 cells/ml) was measured at wavelengths of 339 nm for excitation and 492 nm for emission. Completion of Quin-2 hydrolysis was verified by recording the shift of the peak of maximal fluorescence from 430 to 492 nm. The fluorescence signal was calibrated by adding 4 mM-EGTA/30 mM-Tris base, which decreased extracellular Ca^{2+} concentration to approx. 1 nM. The immediate initial decrease (less than 10 s) of fluorescence upon addition of EGTA was considered to represent Quin-2 hydrolysed externally and was taken into account to correct all values. Minimum fluorescence ($F_{\min.}$) was obtained by lysing the cells with 50 μM -digitonin. After neutralization with HCl, maximum fluorescence ($F_{\max.}$) was recorded by restoring Ca^{2+} concentration to 1 mM. Intracellular Quin-2 concentration was calibrated against a known amount of Quin-2 free acid.

$[\text{Ca}^{2+}]_i$ was determined from the equation:

$$[\text{Ca}^{2+}]_i = K_d \left(\frac{F - F_{\min.}}{F_{\max.} - F} \right)$$

where $K_d = 115$ nM (Tsien *et al.*, 1982).

Intracellular Ca^{2+} stores were evaluated as follows: after 6–8 min incubation in Ca^{2+} -free buffer containing 0.05 mM-EGTA, 1 mM of the bivalent cationophore ionomycin (kindly provided by Dr C. M. Liu, Hoffman-La Roche, Nutley, NJ, U.S.A.) was added. The increase in $[\text{Ca}^{2+}]_i$ observed in these conditions is thought to result from the mobilization of intracellular Ca^{2+} stores. Indeed, the amount of newly formed Ca^{2+} -Quin-2 complex approaches the amount of Ca^{2+} released from internal stores if Quin-2 is in excess of the intrinsic cytoplasmic binding sites of similar affinity. The Ca^{2+} -Quin-2 complex was calculated as shown by Grinstein *et al.* (1984) and Berthon *et al.* (1984), by using the equation:

$$\text{Complex} = \frac{\text{total Quin-2}}{1 + \frac{K_d}{[\text{Ca}^{2+}]_i}}$$

whereas the total cytoplasmic Ca^{2+} was taken as the sum of $[\text{Ca}^{2+}]_i$ and Ca^{2+} -Quin-2 complex.

Other methods

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. SDS (0.5%) was added to the alkaline copper-tartrate solution in order to prevent any precipitate with Lubrol PX. DNA was measured as described by Burton (1956), with calf thymus DNA as standard. Results are given as means $\pm$ S.E.M., and differences were evaluated by a two-tailed Student's *t*-test.

Table 1. Effect of different concentrations of dexamethasone on stimulation by PTH and isoproterenol of cyclic AMP production

Confluent cells were incubated with various concentrations of dexamethasone for the last 2 days in culture. Cyclic AMP production was measured on day 10 after plating. Cells were exposed to 250 nM bovine PTH or to 0.1 mM isoproterenol in the presence of 0.2 mM-IBMX for 7 min. The results are means $\pm$ SEM for triplicate determinations.

[Dexamethasone] (nM)	Cyclic AMP (pmol/7 min per μg of DNA)		
	Control	+PTH	+Isoproterenol
None	3.5 ± 0.7	65.9 ± 1.8	91.5 ± 1.8
1	3.8 ± 0.2	82.1 ± 6.6	98.2 ± 2.2
10	4.2 ± 0.5	105.1 ± 5.7	112.2 ± 5.4
100	4.6 ± 0.6	109.5 ± 4.0	105.4 ± 2.5

Fig. 1. Effect of dexamethasone on cyclic AMP production by intact cells in response to bovine PTH or forskolin

Confluent cells were incubated with 100 nM-dexamethasone ($\circ$, Δ) or ethanol ($\bullet$, $\blacktriangle$) for the last 2 days in culture. Cyclic AMP production in response to various concentrations of PTH ($\bullet$, $\circ$) or forskolin ($\blacktriangle$, Δ) was measured on day 10 after plating in the presence of 0.2 mM-IBMX for 7 min. Results are means $\pm$ S.E.M. for triplicate determinations.

RESULTS

Influence of dexamethasone on cyclic AMP production

Table 1 shows the effect of treatment for 48 h with dexamethasone on PTH- or isoproterenol-stimulated cyclic AMP production in ROS 17/2.8 cells. The steroid caused an enhanced stimulation from 1 nM upwards. The apparent EC_{50} was about 3 nM. The effect was present already after 24 h of incubation with the glucocorticoid and persisted for at least 24 h after its removal (results not shown).

All experiments with intact cells were performed in the presence of 0.2 mM of the phosphodiesterase inhibitor IBMX; at this concentration the effect was maximal.

Fig. 2. Effect of dexamethasone on adenylate cyclase activity

Plasma membranes were isolated on day 10 after plating from cells treated with 100 nM-dexamethasone (□) or ethanol (●) for the last 2 days in culture. The incubation mixture contained 10 μ M-GTP and various concentrations of the different agonists for 10 min. The values are means of triplicate determinations.

Indeed, whereas the PTH-stimulated cyclic AMP production in the absence of IBMX was 67.8 ± 4.2 and 100.0 ± 2.9 pmol/7 min per μ g of DNA ($n = 3$) in ethanol- and dexamethasone-treated cells respectively, these values were 100.6 ± 3.3 and 142.5 ± 2.0 in the presence of 0.2 mM-IBMX, and 102.7 ± 4.9 and 147.9 ± 5.6 in the presence of 2 mM-IBMX. Thus the dexamethasone-induced increase in the cyclic AMP response to PTH was similar in the presence or the absence of the phosphodiesterase inhibitor.

Forskolin is an activator of adenylate cyclase capable of directly stimulating the catalytic unit in intact cells. Fig. 1 shows the dose-response relationship for PTH and forskolin. In dexamethasone-treated cells, the maximal stimulation for both agonists was about 160% of that observed in control cells. Dexamethasone did not influence the agonist concentration producing the half-maximal stimulation.

Influence of dexamethasone on adenylate cyclase activity

To investigate further the possible mechanisms of the observed dexamethasone effect, adenylate cyclase activity

was measured in isolated plasma membranes. This approach allowed us to study not only the hormonal stimulation, but also the activation by agonists acting on the guanine nucleotide regulatory protein (p[NH]ppG, NaF) or on the catalytic unit (forskolin, Mn^{2+}). In plasma membranes isolated from cells treated with 100 nM-dexamethasone for 48 h, the stimulations produced by PTH, isoproterenol, p[NH]ppG or forskolin were markedly enhanced (Fig. 2). The response to NaF was also increased (Table 2).

To test whether dexamethasone enhanced adenylate cyclase activity in non-osteoblastic bone-derived cells, the ROS 24/1 cells, which are devoid of the osteoblastic features shared by ROS 17/2.8 cells (Majeska *et al.*, 1980), were incubated with 100 nM-dexamethasone for 48 h. In plasma membranes isolated from these cells, no stimulation of the response to NaF or forskolin was detected (Table 2).

Similar results were obtained when cyclic AMP production was measured in intact ROS 24/1 cells. In glucocorticoid-treated cells it was 26.6 ± 1.3 pmol/7 min per μ g of DNA (control 29.4 ± 0.7) and 41.4 ± 3.0

Table 2. Effect of dexamethasone on adenylate cyclase activity in plasma membranes from ROS 24/1 and ROS 17/2.8 CELLS

Confluent cells were incubated with 100 nM-dexamethasone or ethanol for the last 2 days in culture. Plasma membranes were isolated on day 10 after plating. Adenylate cyclase activity was measured in the presence of 10 μ M-GTP, 200 nM bovine PTH, 10 μ M-isoproterenol, 10 mM-NaF or 50 μ M-forskolin for 10 min. In parentheses are shown the results of another experiment with a different membrane preparation, where GTP was omitted. The results are means $\pm$ S.E.M. for triplicate determinations.

Cells . . .	Adenylate cyclase activity (pmol/min per mg)			
	ROS 24/1		ROS 17/2.8	
	–	+	–	+
Dexamethasone . . .				
Control	9.3 $\pm$ 0.8 (1.5 $\pm$ 0.5)	10.1 $\pm$ 0.8 (2.6 $\pm$ 1.5)	36.6 $\pm$ 3.2	42.4 $\pm$ 6.0
PTH	9.2 $\pm$ 0.7	10.5 $\pm$ 0.7	253.9 $\pm$ 13.6	571.6 $\pm$ 5.6
Isoproterenol	13.8 $\pm$ 0.9 (4.4 $\pm$ 0.3)	12.6 $\pm$ 1.0 (3.0 $\pm$ 0.7)	399.6 $\pm$ 10.7	599 $\pm$ 4.8
NaF	111.0 $\pm$ 2.9 (42.6 $\pm$ 0.4)	73.6 $\pm$ 3.8 (43.4 $\pm$ 1.1)	612.9 $\pm$ 20.7	883.2 $\pm$ 33.2
Forskolin	52.9 $\pm$ 3.0	53.9 $\pm$ 2.4	179.0 $\pm$ 9.6	276.1 $\pm$ 11.7

Fig. 3. S 49 CYC⁻ reconstituted adenylate cyclase activity reflecting G-unit activities

Lubrol-PX extract of plasma membranes was prepared from control cells (●) or dexamethasone (100 nM)-treated cells (□) for the last 2 days in culture. Various amounts of extract were assayed in triplicate with either 10 mM-NaF or 0.1 mM-p[NH]ppG. The values are means $\pm$ S.E.M.

(control 36.2 $\pm$ 3.4) in response to 5 μ M- and 50 μ M-forskolin respectively. Thus enhancement of the cyclic AMP response induced by dexamethasone appeared to be specific for osteoblast-like cells.

Since the diterpene forskolin is thought to activate directly the catalytic unit of the adenylate cyclase (Seamon & Daly, 1981), our results suggested an increased activity of this component to account for the dexamethasone-induced increment in cyclic AMP production. However, it has been shown that the potency of forskolin is enhanced in the presence of active guanine nucleotide regulatory protein (Downs & Aurbach, 1982; Clark *et al.*, 1982; Darfler *et al.*, 1982). To investigate whether the dexamethasone effect could result from an

increase of the G-unit activity, modulating thereby the response of the catalytic unit to forskolin, G-unit activity was directly measured in a Lubrol-PX extract by using the reconstitution assay. Fig. 3 shows that there was no difference in G-unit activity stimulated by either NaF or p[NH]ppG in plasma membranes isolated from dexamethasone- or ethanol-treated cells. Similar results were obtained with a cholate extract (results not shown). Thus the dexamethasone effect did not appear to result from a change in the G-unit, but more likely from an involvement of the catalytic unit itself. A further argument in favour of this hypothesis was provided by using Mn²⁺-ATP as substrate, since Mn²⁺ can directly activate the catalytic unit. As shown in Table 3, the

stimulation of adenylate cyclase caused by Mn^{2+} was markedly higher in plasma membranes isolated from glucocorticoid-treated cells.

Finally, to determine whether glucocorticoid could change the sensitivity to Mg^{2+} and alter thereby the response to PTH, the effect of various Mg^{2+} concentrations was studied. The results presented in Fig. 4 indicate that

Table 3. Effect of dexamethasone on Mn^{2+} stimulation of adenylate cyclase activity

Confluent cells were incubated with dexamethasone or ethanol for the last 2 days in culture. Plasma membranes were isolated on day 10 after plating. Adenylate cyclase activity was measured by incubating the plasma membrane with various concentrations of Mn^{2+} for 10 min. Values are means $\pm$ S.E.M. for triplicate determinations.

[Mn^{2+}] (mM)	Adenylate cyclase activity (pmol/min per mg)	
	Ethanol	Dexamethasone
2	116.2 $\pm$ 1.9	189.9 $\pm$ 1.8
5	192.2 $\pm$ 10.5	296.8 $\pm$ 4.7
10	262.4 $\pm$ 7.1	430.3 $\pm$ 10.1

Fig. 4. Regulation of PTH stimulation of adenylate cyclase by Mg^{2+}

Confluent cells were incubated with 0.1% ethanol (○) or 100 nM-dexamethasone (▲) for the last 2 days in culture. Plasma membranes were isolated on day 10 after plating. Adenylate cyclase activity was measured as described in the Materials and methods section with various concentrations of Mg^{2+} . The results represent the stimulation obtained with 200 nM-PTH minus the basal value.

dexamethasone increased the maximal velocity of the enzymic reaction, and not the Mg^{2+} concentration producing half-maximal effect.

Influence of dexamethasone on cellular Ca^{2+} metabolism

When ROS 17/2.8 cells were loaded with 100 μ M-Quin-2 acetoxymethyl ester, about 40% of the latter was hydrolysed externally, as assessed by the immediate fall in fluorescence detected upon addition of 4 mM-EGTA/30 mM-Tris base. In these conditions, the concentration of Quin-2 trapped intracellularly was 1–2 mmol/litre of cell water {by using 3H_2O and [^{14}C]poly(ethylene glycol) as extracellular marker (Rotenberg, 1979), cell water was found to be 1.50 and 1.86 μ l/ 10^6 cells in ethanol- and dexamethasone-treated cells respectively}. Cell autofluorescence accounted for about 20% of the fluorescence signal. In the presence of 1 mM extracellular Ca^{2+} ($[Ca^{2+}]_o$), $[Ca^{2+}]_i$ was 143 ± 15 nM ($n = 6$). The bivalent cationophore ionomycin (1 μ M) produced a 3.4 ± 0.2 -fold increase in $[Ca^{2+}]_i$, but 10 nM–1 μ M of either bovine PTH-(1–84)-peptide or bovine PTH-(1–34)-peptide did not affect $[Ca^{2+}]_i$. Similarly, cell depolarization by adding 30 mM-KCl did not change $[Ca^{2+}]_i$, ruling out the existence of voltage-dependent Ca^{2+} channels in osteoblast-like cells.

In several cell systems, the addition of ionomycin in the absence of extracellular Ca^{2+} produces a rise in $[Ca^{2+}]_i$, which is thought to represent the mobilization by the ionophore of Ca^{2+} from intracellular stores (Berthoin *et al.*, 1984; Grinstein *et al.*, 1984). By measuring $[Ca^{2+}]_i$ before and after ionomycin addition as well as the amount of the Ca-Quin-2 complex, the size of these stores was evaluated.

Table 4 shows the influence of dexamethasone on $[Ca^{2+}]_i$ and ionomycin-releasable stores. In the absence or presence of 1 mM- $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ was not significantly altered by dexamethasone treatment. From the changes in $[Ca^{2+}]_i$ induced by ionomycin when the cells were in a Ca^{2+} -free buffer (Fig. 5), intracellular Ca^{2+} stores were determined and found to be decreased in steroid-treated cells.

DISCUSSION

In the osteoblast-like rat osteosarcoma-cell line ROS 17/2.8, the glucocorticoid dexamethasone led to a marked increase in the cyclic AMP response to PTH, as previously shown in transformed and non-transformed bone cells (Chen & Feldman, 1978, 1979, 1984; Rodan *et al.*, 1984; Catherwood, 1985) and in bone organs (Hahn & Halstead, 1979; Ng *et al.*, 1979). This stimulatory effect was not specific for the Ca^{2+} -regulating

Table 4. Effect of dexamethasone on $[Ca^{2+}]_i$ and on ionomycin-releasable intracellular Ca^{2+} stores

Cells were treated and intracellular Ca^{2+} stores measured as described in the Materials and methods section. The results are means $\pm$ S.E.M. for the numbers of determinations in parentheses. Abbreviation: NS, not significant

	$[Ca^{2+}]_o$	Ethanol	Dexamethasone	
$[Ca^{2+}]_i$ (nM)	1 nM	50.2 $\pm$ 5.2 (14)	60.5 $\pm$ 7.5 (11)	(NS)
	1 mM	119.9 $\pm$ 13.2 (12)	95.0 $\pm$ 31.6 (6)	(NS)
Ca^{2+} stores (pmol/ 10^6 cells)		146.4 $\pm$ 22.5 (11)	65.1 $\pm$ 23.5 (9)	($P < 0.025$)

Fig. 5. Influence of dexamethasone on the $[Ca^{2+}]_i$ response to ionomycin in Ca^{2+} -free buffer

Confluent cells were exposed to 0.1% ethanol or 100 nM-dexamethasone for the last 2 days in culture. Preparation and loading of the cells with the fluorescent indicator Quin-2 were as described in the Materials and methods section. After 6–8 min in Ca^{2+} -free buffer containing 0.05 mM-EGTA, 1 μ M-ionomycin (arrow) was added.

hormone PTH, since it was also observed in response to the β -adrenergic agonist isoproterenol confirming findings by Rodan *et al.* (1984).

Since the adenylate cyclase system is a multi-component complex, it was decided to investigate whether one of the different components could be specifically affected by dexamethasone treatment. The responses observed to agonists known to act on different levels of the complex were compatible with the hypothesis that the activity of the catalytic unit itself was enhanced by glucocorticoid. Indeed, the dexamethasone-induced enhancement of the cyclic AMP stimulation was observed not only with agents acting on the G-unit, such as the non-hydrolysable analogue of GTP (p[NH]ppG) or NaF, but also with agonists known to influence directly the catalytic unit such as the diterpene forskolin or the cation Mn^{2+} . However, in other systems the response to forskolin appears to be modulated by the presence of an active G-unit, since the forskolin-stimulated cyclic AMP production is much lower in the CYC⁻ clone, which is genetically devoid of this component, than in the wild-type S49 lymphoma cells, which possess a fully active G-unit (Downs & Aurbach, 1982; Clark *et al.*, 1982; Darfler *et al.*, 1982). Thus to investigate whether the increased cyclic AMP response in dexamethasone-treated cells could be due to a higher G-unit activity, we measured this activity by reconstitution of membrane extract into CYC⁻ membranes. No difference in G-unit activity isolated from cells incubated either with dexamethasone or with the ethanol vehicle was detectable. Moreover, the dexamethasone-induced increase in cyclic AMP in response to Mn^{2+} indicated that the activity of the catalytic unit itself was enhanced in glucocorticoid-treated cells. Similarly, a higher response to forskolin and Mn^{2+} were arguments against the possibility of dexamethasone acting by a deinhibition of a guanine nucleotide binding protein inhibitory influence.

The half-maximal effect of dexamethasone was observed at a concentration similar to the affinity for glucocorticoid, as assessed by radioreceptor studies (Haussler *et al.*, 1980). Moreover, not only was this effect detectable in isolated membranes, but it persisted after removal of dexamethasone. These results strongly suggest a receptor-mediated process, and rule out a direct

chemical interaction between the steroid hormone and the cell membrane.

A soluble cytoplasmic factor increasing adenylate cyclase activity has been described in several cells (Egan *et al.*, 1980; MacNeil *et al.*, 1980; Sahyoun *et al.*, 1983). The cyclase-stimulatory influence of dexamethasone thus could result from a larger amount of this factor. However, this possibility seems to be unlikely, since the dexamethasone-induced effect was detectable in isolated membranes even after extensive washing.

Similarly, the higher cyclic AMP production in glucocorticoid-treated cells could be due to the inhibition of the phosphodiesterase (Manganiello & Vaughan, 1972). If this inhibition plays a role, at least in intact cells, then it would be very unlikely to account for all the steroid-induced stimulatory effect. Moreover, consistent with the findings by Ng *et al.* (1979) and Rodan *et al.* (1984), we observed a higher cyclic AMP response in dexamethasone-treated cells, even in the presence of saturating concentration of the phosphodiesterase inhibitor IBMX.

Abnormal cellular Ca^{2+} distribution has been reported in glucocorticoid-treated cells (Rasmussen & Gustin, 1978; Hällgren *et al.*, 1985), and altered cellular Ca^{2+} metabolism has been suggested to play a role in the glucocorticoid-induced enhancement of adenylate cyclase activity by an unknown mechanism (Chen & Feldman, 1984). We investigated various aspects of cell Ca^{2+} by using the intracellular fluorescent indicator Quin-2. In the presence of 1 mM- $[Ca^{2+}]_o$, the resting $[Ca^{2+}]_i$ was about 140 nM. $[Ca^{2+}]_i$ was sensitive to $[Ca^{2+}]_o$, because, when the latter was decreased to about 1 nM, $[Ca^{2+}]_i$ was 50–60 nM. As reported by Menczel & Zimlichman (1985), no change in $[Ca^{2+}]_i$ could be detected after addition of PTH, in conditions where both PTH stimulation of cyclic AMP production and binding of radioiodinated PTH were observed (the present results; Rizzoli *et al.*, 1983). Whereas resting $[Ca^{2+}]_i$ was not changed, intracellular Ca^{2+} stores were significantly decreased in glucocorticoid-treated cells. The ionomycin-mobilized Ca^{2+} stores which participate in immediate changes in $[Ca^{2+}]_i$ are thought to be associated with the inner face of the plasma membrane and endoplasmic reticulum (Grinstein *et al.*, 1984; Berthon *et al.*, 1984). They certainly represent an

underestimation of the total cellular Ca^{2+} stores. The interpretation of our observation would be highly speculative, as its relationship with the altered activity of adenylate cyclase catalytic unit observed in glucocorticoid-treated cells. Therefore these aspects require further investigations.

In conclusion, osteoblast-like cells incubated with dexamethasone exhibited increased cyclic AMP synthesis in response to agonists acting on the different components of the hormone receptor-guanine nucleotide regulatory unit-adenylate cyclase complex. The results indicated that the activity of the catalytic unit was increased in these cells and could account for these effects. Moreover, the intracellular Ca^{2+} stores mobilizable by ionomycin were decreased in dexamethasone-treated cells.

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