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POSSIBLE MECHANISMS FOR THE PERMISSIVE ACTION OF GLUCOCORTICOID HORMONES: STUDIES ON CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY OF RAT LIVER AND OF MOUSE L1210 CELLS

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SUMMARY

mechanisms for this permissive action were examined: a change in activity of cyclic AMP-dependent protein kinase and an inhibition of the thermostable protein inhibitor of protein kinase.

In rat liver, neither protein kinase activity, nor its stimulation by cyclic AMP was modified following adrenalectomy, corticoid administration or *in vitro* addition of receptor–dexamethasone complex. Moreover, under these conditions, activity of the thermostable protein inhibitor remained unchanged. Similarly negative results were obtained in corticoid-responsive mouse L1210 cells.

Thus, at least in these systems, the hypothesis is not verified. It remains possible that, in the intact cell, corticoids act on cyclic AMP-dependent events that are not accessible to our experimental conditions *in vitro*.

INTRODUCTION

The presence of glucocorticoids is necessary for full expression of a number of responses to other hormones. In fed adrenalectomized rats possessing sufficient hepatic glycogen, the hyperglycemic effect of epinephrine is depressed unless cortisol is administered [1]. Glucocorticoids are also required for epinephrine-induced glycogenolysis in perfused heart [2] and liver [3]. The impaired gluconeogenic response of perfused liver to epinephrine or glucagon in adrenalectomized rats is restored after perfusion of glucocorticoids [3]. In kidney cortex explants from adrenalectomized rats, vasopressin-induced gluconeogenesis is enhanced by dexamethasone [4]. Activation of lipolysis by catecholamines and ACTH *in vitro* is also reduced in fat cells from adrenal-deficient rats [3]. In amphibian epithelia, vasopressin-induced increased water permeability can be enhanced by glucocorticoids [5]. Finally, glucagon-stimulated amino acid uptake by isolated hepatocytes is increased by dexamethasone [6].

The mechanism of this permissive [7] action of glucocorticoids remains obscure. It is thought that the effects of the aforementioned non-steroid hormones result from their binding to a membrane receptor followed by activation of adenylate cyclase, accumulation of cyclic AMP[1] and stimulation by the nucleotide of protein kinase. This protein consists of an inactive holoenzyme made of regulatory and catalytic subunits. Binding of cyclic AMP to the regulatory subunit liberates free catalytic subunits capable of catalyzing the phosphorylation, from ATP, of specific proteins engaged in the hormonal effect [8]. In principle, any of these steps could be under glucocorticoid control. However, when the cyclic AMP-dependent hormonal effect is mimicked by cyclic AMP itself, this is also impaired by adrenalectomy [1, 3, 4]. In addition, basal [9] and hormonally-induced [2] cyclic AMP levels are normal in target tissues of adrenalectomized animals. It is therefore likely that glucocorticoids exert their permissive action by influencing some event beyond the generation of cyclic AMP, for instance, the activity of cyclic AMP-dependent protein kinase. We have tested this hypothesis in rat liver and in mouse leukaemia L1210 cells.

[1] *Abbreviations*: cyclic AMP, adenosine 3′:5′-monophosphate; protein kinase, cyclic AMP-dependent ATP: protein phosphotransferase, EC 2.7.1.37; dexamethasone, 9α-fluoro-11β, 17α, 21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione; HSPI, heat-stable protein kinase inhibitor.

MATERIALS AND METHODS

Animals. Male Wistar rats (150–250 g) fed a standard laboratory diet were fasted for 18 h except for experiments reported under Results Al, and were killed by decapitation. When adrenalectomized, they received 0.9% NaCl in drinking water. Adrenalectomy was considered effective when plasma corticosteroids determined by competitive-binding assay [10] were undetectable at the time of experiment, three days or more after surgery. Cortisol treatment consisted in one intraperitoneal injection of 5 μg per g body weight, 90–120 min prior to sacrifice.

Cell cultures. L1210 cells, an established line of mouse leukaemia kindly provided by Dr. I. Gresser, were grown in suspension in RPMI 1640 (Gibco) containing 10% foetal calf serum. They were resuspended in serum-free medium containing 0.1% BSA with or without 1 μM dexamethasone, a gift from Merck, Sharp & Dohme. After 16 h, cells were washed with phosphate-buffered saline and homogenized by sonication in 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA.

Glucocorticoid-receptor binding. Rat liver was homogenized in one vol. of ice-cold buffer containing 250 mM sucrose, 2 mM dithiothreitol (CalBiochem) and 10 mM Na–K–phosphate, pH 7.4. Cytosol was obtained by centrifuging the homogenate at 800 *g* for 10 min, followed by centrifugation of the supernate at 200,000 *g* for 60 min at 0°C. Concentration of glucocorticoid receptor sites in cytosol and in nuclear fraction was determined as described earlier [11, 12].

Preparation and assay of HSPI. Liver was homogenized in 10 mM Tris–HCl, pH 7.5, and HSPI, prepared as described by Appleman *et al.*[13], was dialyzed against 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA. Protein concentration of HSPI resuspended in 1 ml buffer per g tissue was 3 mg per ml, achieving a 75-fold purification which is identical to that reported by Walsh *et al.*[14]. The activity of HSPI was determined from its inhibitory action on cyclic AMP-activated protein kinase purified from bovine muscle by the simplified method of Gilman[15].

Protein kinase activity. This was determinded as described elsewhere [16] using as exogenous substrate calf thymus histone type II-A (Sigma) for rat liver protein kinase and type f_2b (Sigma VII-S) for L1210 cells and for muscle protein kinase, the reference for assaying HSPI. Basal and total activities are those measured in absence and presence of cyclic AMP, respectively. Protein kinase activity of L1210-R cells was determined on the 27,000 *g* (30 min) supernatant fraction of a homogenate containing 0.15 M NaCl, while that of L1210-SW cells was determined after further centrifugation of this supernatant at 105,000 *g* for 30 min. This difference in methodology accounts for the apparent difference in specific activity of cyclic AMP-dependent protein kinase between both cell lines (see Fig. 4). Also, in the case of L1210 cells, phosphate incorporation by endogenous substrate was subtracted from values obtained in presence of added histone.

RESULTS AND DISCUSSION

A. *Studies on rat liver*

(1) *Lack of direct effect of dexamethasone on protein kinase activity.* Physiological effects of glucocorticoids appear to require binding of these hormones to a specific intracellular receptor protein [reviewed in Ref. 17]. Protein kinase activity of rat liver cytosol was therefore determined after allowing cell-free binding of dexamethasone (50–200 nM) to its receptor in unfractionated homogenate. In 8 experiments, concentration of bound receptor was 0.36 ± 0.03 S.E.M. and 0.55 ± 0.06 S.E.M. pmol per mg cytosol protein in control and adrenalectomized rat liver, respectively. This binding had no effect on protein kinase activity in cytosol from adrenalectomized rats (Table 1). Similarly negative results were obtained upon binding dexamethasone to cell-free cytosol (not shown).

Table 1. Lack of effect of receptor-dexamethasone binding on protein kinase activity of rat liver cytosol

n	activation	basal kinase	total kinase
2	–	106	104
5	+	110	101

Liver homogenates from adrenalectomized rats were incubated for 90 min at 0°C in the presence of 50–200 nM dexamethasone. Activation of receptor–dexamethasone complex consisted in raising temperature to 20°C and adding NaCl to 0.15 M during the last 30 min of incubation [18]. Cytosol was prepared from these homogenates and protein kinase activity was determined in absence (basal kinase) and presence (total kinase) of cyclic AMP. Results are in mean percent of cytosol protein kinase from matched homogenate aliquots similarly incubated, but in the absence of dexamethasone. n refers to number of experiments.

In cell-free systems, receptor-steroid complexes can be "activated" by raising ionic strength to 0.15–0.30 and (or) temperature above 0°C. This would allow receptor–steroid complex to interact with the cell nucleus, a presumably important step in corticoid action [18]. Thus, dexamethasone-bound homogenate was treated with 0.15 M NaCl for 30 min at 20°C, upon which steroid became specifically bound (1.0 pmol per mg nuclear protein) to the nuclear fraction from that homogenate, concomitant with a 70% decrease in cytosol receptor concentration. Under these conditions, basal protein kinase activity of cytosol now increased to about twice the value obtained in the absence of dexamethasone, while kinase activity in the homogenate remained unaltered. However,

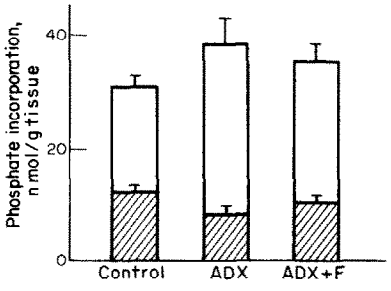

Fig. 1. Rat liver protein kinase activity following adrenalectomy and cortisol treatment. Phosphate incorporation into exogenous histone was determined in absence (lower portion of bar graphs) and presence (whole bar graph) of 2.5 μM cyclic AMP. Results are means ± S.E.M. of 15 experiments on control rats and of 16 experiments on adrenalectomized (adx) rats, half of which had received cortisol (F) as described in Methods.

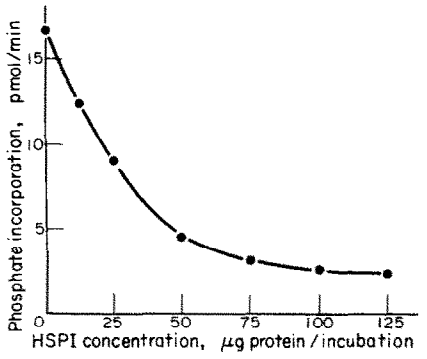

Fig. 2. Titration of Rat Liver HSPI. Phosphate incorporation into histone, catalyzed by bovine muscle protein kinase in the presence of cyclic AMP without and with increasing concentrations of rat liver HSPI.

these effects could be reproduced by "activating" homogenate even in the absence of steroid. In fact, increased ionic strength released catalytic subunits of protein kinase adsorbed to particles in the homogenate thereby artifactually increasing the specific activity of protein kinase in cytosol from that homogenate [for a discussion, see ref. 16]. Using the proper salt-treated controls it became apparent that activation of dexamethasone-bound receptor did not alter basal or total kinase activity in the cytosol (Table 1).

(2) *Protein kinase activity in liver from adrenalectomized and cortisol-treated rats.* Since dexamethasone, either free or bound to its receptor, had no direct effect on protein kinase activity *in vitro*, we examined whether this activity was modified after *in vivo* alterations in the adrenal status. As shown in Fig. 1, basal protein kinase activity was unchanged following adrenalectomy without or with cortisol treatment. Moreover, total protein kinase activity did not change, indicating that cyclic AMP could stimulate the enzyme to the same extent in liver from adrenal-deficient as from normal rats. Likewise, cortisol treatment had no effect on the stimulatory action of cyclic AMP on protein kinase activity. Despite this intact sensitivity of the enzyme to the nucleotide *in vitro*, other factors known to influence protein kinase *in vivo* might be altered following adrenalectomy. One such factor is the so-called heat-stable protein inhibitor (HSPI) of protein kinase [13, 14].

(3) *Activity of rat liver HSPI after adrenalectomy and cortisol treatment.* There is enough HSPI in the cell to prevent expression of a substantial proportion of cyclic AMP-stimulated protein kinase activity *in vivo* [19]. Therefore, we determined whether adrenalectomy increased HSPI concentration in rat liver. HSPI was titrated by its inhibitory action on histone phosphorylation catalyzed by exogenous protein kinase (Fig. 2). HSPI concentration (680 units per g tissue) was identical to that reported by others for rabbit liver [20]. It can be seen from Fig. 3 that adrenalectomy had no effect on the activity of rat liver HSPI. Moreover, the activity of the inhibitor remained unaltered following cortisol treatment of either normal or adrenalectomized rats.

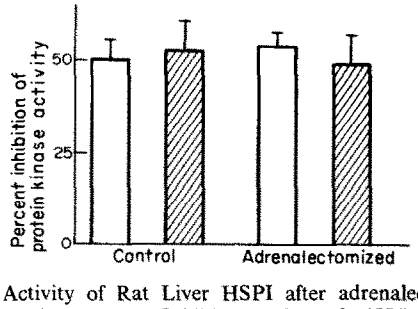

Fig. 3. Activity of Rat Liver HSPI after adrenalectomy and cortisol treatment. Inhibitory effect of HSPI (30 μg protein per incubation) on muscle protein kinase activity determined as in Fig. 2. Results are means ± S.E.M. of separate experiments on individual HSPI preparations from 10 control and 12 adrenalectomized rats, half of which had been treated with cortisol (shaded blocks) as described.

B. *Protein kinase activity of mouse leukaemia L1210 cells*

Given these negative results, it was worth testing our hypothesis in cultured cells, where steroid concentrations can be controlled much better than in whole animals. Two clones of corticoid-responsive L1210 cells were studied. The first clone, L1210-R is growth-inhibited by dexamethasone [21]. The cells were resuspended in serum-free medium in order to remove any steroid present in the culture medium. Parallel cultures treated similarly received 1 μM dexamethasone. After 16 h, protein kinase activity was determined (Fig. 4). Again, there was no effect of dexameth-

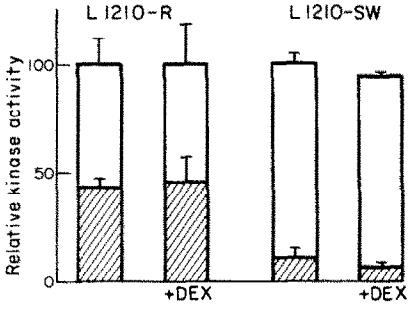

Fig. 4. Protein kinase activity in mouse L1210 cells. Phosphate incorporation into exogenous histone was determined in absence (lower portion of bar graphs) and presence (whole bar graph) of 2.5 μM cyclic AMP. Results, in percent of steroid-free control, are means ± S.E.M. of 8 and 4 experiments with L1210-R and L1210-SW cells, respectively, half of which conducted in presence of dexamethasone (DEX) as described. Absolute values for total kinase in control cultures were 161 and 666 pmole phosphate incorporated per mg protein per min for L1210-R and L1210-SW cells, respectively. The higher S.A. in the latter is not related to the cell type but is due to the higher degree of purification of cyclic AMP-dependent protein kinase over whole homogenate in these particular experiments (see Methods).

asone on either basal or cyclic AMP-stimulated phosphorylation of exogenous histone.

Another clone L1210-SW was then examined in which a permissive action of corticoids can be demonstrated. These cells are growth-inhibited by low, and otherwise ineffective, concentrations of mouse interferon providing dexamethasone is present. Dexamethasone alone does not affect the growth of these cells (unpublished observations).

Since cyclic AMP concentration increases in mouse L cells treated with an interferon preparation [J. Wérenne, A. Laruel and R. Laruel, unpublished; and ref. 22] we verified whether the sensitivity of protein kinase to cyclic AMP was modified by dexamethasone in these cells. This was not the case (Fig. 4).

In conclusion, these experiments do not support the idea that in rat liver the activity of cyclic AMP-dependent protein kinase is under the control of glucocorticoid hormones. Similar results have been published by Friedmann[23] since our studies have been completed. This is in contrast with the reported stimulatory effect of dexamethasone on cyclic AMP-dependent protein kinase activity of rat H-4-11-E hepatoma cells [24] and epididymal fat pads [25]. Our negative findings extend to two glucocorticoid-responsive mouse leukaemia cell lines. Obviously, if glucocorticoids favour expression of the effects of cyclic AMP in the systems studied here, it may be through factors that influence protein kinase *in vivo* but cannot be detected in our *in vitro* assay, or else it may be on a limiting step beyond activation of protein kinase.

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DISCUSSION

Munck. Is there any reason to suppose that protein kinase is involved in the effect of interferon in cell killing and that therefore the so-called permissive effect may involve protein kinase?

Rousseau. No, the only evidence we have so far is that cyclic AMP increases in cells treated with interferon (Weber and Stewart, *J. Gen. Virol.* **28** (1975) 363; Wérenne, unpublished). However, it is not established that this bears a causal relationship to interferon action. This is currently investigated.

Munck. Is it possible that the cyclic AMP hypothesis of permissive action is a red herring, and that it may be better to look elsewhere?

Rousseau. I agree.