A Radioautographic Study on the Mechanisms by which Adrenal Glucocorticoids Play their Permissive Role in the Biological Activity of Cyclic AMP in the Liver Cell

Glucocorticoid hormones have been reported to amplify the effects of adenosine 3′,5′-cyclic monophosphate (cAMP) on glycogen metabolism[1-3], gluconeogenesis[1], and enzyme induction in liver tissue and hepatoma cells in vivo and in vitro[4-8]. The operative mechanisms of such a 'permissive' role of glucocorticoids remain as yet rather obscure[9]. We aimed, therefore, at further elucidating them by investigating the mutual effects of a prior exposure to either a glucocorticoid hormone or cAMP on the macromolecular binding of, respectively, cAMP-H^3 or cortisol-H^3 by rat primary hepatic cells in culture.

Materials and methods. The livers, aseptically drawn from 10–16 Wistar SM albino rats aged 6 days belonging to the same littermate, were pooled and cultured as previously communicated[10], except for the addition to the Hanks'-BSS-trypsin solution (0.25% w/v) of hyaluronidase type II and collagenase type I (both at 0.02% w/v, Sigma). This modification allowed the simultaneous cultivation of hepatocytes both as single dispersed cells and as more or less wide, one-cell-thick, sheets (Figure 1). In this work, 2 groups of 32 separates were used. On the 4th day in vitro 16 specimens of each set were treated after either of the following schedules: A) exposure for 24 h to Δ^1-cortisol acetate (10 μg/ml: 2.52×10^{-5} *M*, Schering) followed by pulse (1 h) labelling at 35 °C with cAMP-H^3 (G) ammonium salt (1.0 μCi/ml; specific activity 20 Ci/m*M*; NEN Chemicals GmbH) and then by a thorough washing in PBS (Oxoid) and subsequent fixation in methanol-acetic mixture (9/1 v/v) for 10 min.; B) treatment for 24 h with cAMP (50 μg/ml: 1.51×10^{-4} *M*, Sigma) ensued by 1 h incubation at 35 °C with hydrocortisone-1, 2, 6, 7-H^3(N) (1.0 μCi/ml; specific activity 80 Ci/m*M*; NEN Chemicals GmbH) dissolved in the growth medium in the presence of 40 μg/ml NN-dimethylformamide. The specimens were afterwards fixed in 10% neutral formalin[11]. The remaining 16 separates of each stock served as parallel controls and were labelled as the treated ones. Both control and treated preparations were processed for radioautography in a single batch as already reported[12]. Non-labelled cultures were coated in parallel to estimate the level of background, which was always found to be less than 1 silver grain/100 μm^2 (from 0.2 to 0.6). Developed preparations were stained with haematoxylin-eosin and observed under a Leitz Orthoplan® microscope. Positive and negative chemographic phenomena, which were looked for, were practically absent. The

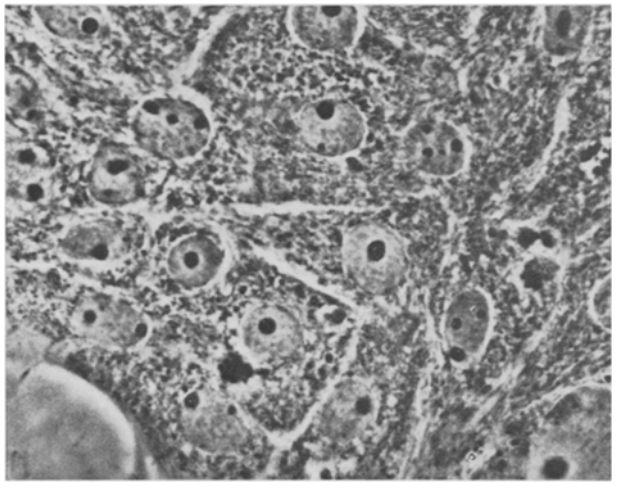

Fig. 1. Monolayer of rat primary hepatic cells in culture on the 4th day in vitro. Cells attached to the polyethylene disc were fixed in glutaraldehyde (2.5% w/v in Sorensen's phosphate buffer) for 30 min at 0 °C, washed in the same buffer, dehydrated and stained with uranyl acetate (saturated solution in absolute alcohol) for 1 h at 0 °C, then washed again in alcohol, left 1 h in phosphotungstic acid (1% w/v in absolute alcohol) at 0 °C, washed in alcohol, clarified in xylene and mounted with DPX. Observation was carried out under phase contrast at ×1,200. The richness in subcellular structures of the hepatocyte cytoplasm is well maintained in vitro.

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Table I. Effect of cAMP (50 μg/ml/24 h) on the uptake of cortisol-1,2,6,7-H^3 by rat primary liver cells

Cell type	Treatment	No. of cells counted	Nucleolus	Nucleoplasm	Total nucleus	Cytoplasm	Total cell
Hepatocyte	C	50	3.58 ± 0.21	9.04 ± 0.41	12.62 ± 0.46	26.80 ± 1.15	39.42 ± 1.37
	cAMP	50	4.90 ± 0.26	9.04 ± 0.43	13.94 ± 0.58	23.66 ± 0.88	37.60 ± 1.14
	Δ%		+36.87[c]	0.00[a]	+10.45[b]	−11.72[b]	−4.62[a]
Fibroblast-like cells	C	50	2.86 ± 0.19	7.54 ± 0.36	10.40 ± 0.38	18.38 ± 0.77	28.78 ± 0.93
	cAMP	50	3.00 ± 0.22	7.26 ± 0.31	10.26 ± 0.40	16.94 ± 0.68	27.20 ± 0.95
	Δ%		+4.89[a]	−3.72[a]	−1.35[a]	−7.84[a]	−5.49[a]

The figures express mean grain counts ± S.E. Δ%: % change in grain count with respect to control values. Level of significance: [a] *P* not significant; [b] $P < 0.01$; [c] $P < 0.001$. C, controls.

Table II. Effect of Δ^1-cortisol (10 µg/ml/24 h) on the uptake of cAMP-H^3 by rat primary liver cells

Cell type	Treatment	No. of cells counted	Nucleolus	Nucleoplasm	Total nucleus	Cytoplasm	Total cell
Hepatocyte	C	50	5.04 ± 0.42	13.46 ± 0.86	18.50 ± 1.22	21.52 ± 1.00	40.02 ± 1.58
	Δ^1-cort.	51	4.31 ± 0.31	10.19 ± 0.56	14.50 ± 0.72	27.90 ± 1.52	42.40 ± 1.97
	Δ%		−14.49[b]	−24.30[d]	−21.63[c]	+29.64[d]	+5.94[a]
Fibroblast-like cells	C	50	4.26 ± 0.31	10.90 ± 0.48	15.16 ± 0.63	16.86 ± 0.72	32.02 ± 1.16
	Δ^1-cort.	50	2.42 ± 0.25	8.30 ± 0.50	10.72 ± 0.58	17.54 ± 1.12	28.26 ± 1.38
	Δ%		−43.20[d]	−23.86[d]	−29.29[d]	+4.03[a]	−11.75[c]

The figures express mean grain counts ± S.E. Δ%: % change in grain count with respect to control values. Level of significance: [a] P not significant; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$. C, controls.

specificity of the labelling could be shown by parallel experiments in which cultured rat liver cells were incubated with: 1. cAMP-H^3 (1.0 µCi/ml) plus 'cold' cAMP (1 mg/ml); 2. cortisol-H^3 (1.0 µCi/ml) plus 'cold' cortisol (400 µg/ml). In both instances a reduction up to 90% of the labelling, with respect to experiments without carriers, could be detected. Since cAMP-H^3 and cortisol-H^3 were incorporated by all cell types, single cells were randomly photographed at ×1200 and silver grains per cell counted. The means were compared by the Student's t-test (P was considered as significant only if < 0.05).

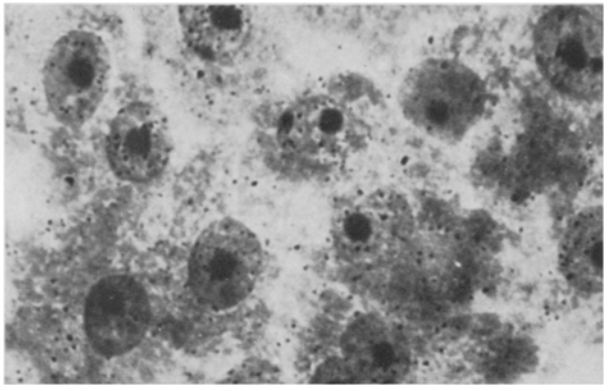

Fig. 2. Rat primary hepatocytes of control cultures labelled with cAMP-H^3. Haematoxylin-eosin. ×1000.

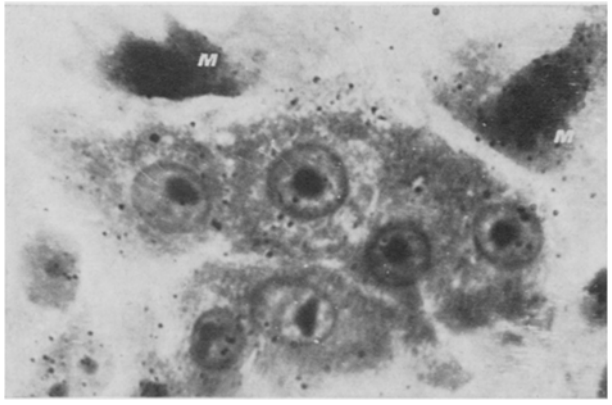

Fig. 3. An islet of rat hepatocytes labelled with cAMP-H^3 after a previous 24 h incubation with Δ^1-cortisol (10 µg/ml). Nuclear, but not cytoplasmic labelling is reduced. A couple of macrophages (M) are seen in the upper part of the field. Haematoxylin-eosin. ×1000.

Results and discussion. We reported previously that primary rat liver cells retain specific ultrastructural features[13] and respond to the enzyme-inducing action of phenobarbital in a way similar to in vivo liver cells[14]. Studies by immunofluorescent methods have shown that cultured hepatocytes actively synthesize a number of rat serum proteins for up to 25 days in vitro[15]. As the data of Table I demonstrate, the pretreatment with cAMP augmented in primary hepatocytes the nucleolar labelling by cortisol-H^3; nucleoplasmic labelling was unchanged, whereas cytoplasmic binding was slightly but significantly reduced. Total cortisol-H^3 uptake did not differ from that of control cells. No change was detected in the labelling of fibroblast-like cells. These findings suggest that cAMP exerts limited effects on the uptake and intracellular disposal of protein-bound cortisol-H^3 by primary rat hepatocytes, since NN-dimethyl-formamide should have extracted from the cells the unbound cortisol (and/or metabolites)[15]. Intracellular dislocation of the macromolecule-bound steroid should have been prevented by the fixation method employed, which causes a minimal loss (1%) of cortisol-H^3 from liver tissue[11]. The transport of protein-bound cortisol into the nucleus, followed by binding of the complex to specific sites of chromatin is believed to precede the transcriptive effects of the hormone[16]. Our results support the view cAMP may facilitate the known[17] enhancing effect of cortisol on the nucleolar RNA polymerase activity. The data reported in Table II show that a previous exposure to Δ^1-cortisol decreased the labelling by cAMP-H^3 over nucleoli and karyoplasm of rat hepatocytes in culture, whilst the cytoplasmic labelling was significantly incremented (Figures 2 and 3). However, total cAMP-H^3 uptake did not significantly differ from that of control cells. Nuclear uptake of cAMP-H^3 was decreased by Δ^1-cortisol also in fibroblast-like cells, but cytoplasmic labelling was not affected; total uptake in these cells was slightly dimin-

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ished. Our findings would suggest that Δ^1-cortisol may impair the intranuclear transport of macromolecule-bound cAMP-H^3 and thereby lessen, but not suppress, the transcriptive effects of the cyclic nucleotide on rat liver chromatin[15,18,19]. On the other hand, it appears reasonable to admit that corticosteriods may act also by inhibiting in part (at the level of transcription?) the synthesis of nuclear macromolecular acceptors of cAMP-H^3, and by simultaneously enhancing the synthesis of a number of cytoplasmic phosphokinases which are activated by the binding of cAMP to their regulatory subunits[20]. Such a different regulation by corticosteroids of the synthesis of nuclear and cytoplasmic cAMP acceptors is supported: 1. by the finding that in hepatocytes the cytoplasmic increment of specific cAMP binding macromolecular sites is percentually greater than their intranuclear simultaneous decrease; 2. by the behaviour of fibroblast-like cells, in which only the nuclear uptake is decreased. A report stating that glucocorticoids do not alter the uptake of cAMP-H^3 by H4-11E hepatoma cells in tissue culture[6] does not contrast with our findings, since hepatoma cells are metabolically different from normal liver cells[6]. The relief of a post-transcriptional role of cAMP has been stressed in several reports[9]. CAMP has been shown to enhance the phosphorylation of ribosome proteins[21], and to influence polypeptide synthesis[22], assembly[23] and release[23,24] at the level of this organelle. Moreover, cAMP has been postulated to modulate, at the level of mRNA translation, adrenal[25], adenohypophyseal[26] and thyroid[27] protein synthetic processes. A microsomal protein, strongly binding cAMP, has been indicated as an essential factor in the induction by cAMP of the release of enzymes from rat liver polysomes[23]. In this frame, our results suggest that glucocorticoid hormones increase in the hepatocyte the cytoplasmic utilization (and thereby metabolic effects[1-5,7]) of cAMP.

Riassunto. I meccanismi che operano alla base dell'effetto «permissivo» dei corticosteroidi sulle azioni metaboliche dell'adenosin-monofosfato-3′,5′-ciclico (cAMP) sono stati indagati in colture primarie di fegato di Ratto. I risultati ottenuti indicano che i glucocorticoidi, mentre diminuiscono l'utilizzazione del cAMP nel nucleo degli epatociti, la aumentano a livello del citoplasma. Viene avanzata l'ipotesi che gli steroidi inibiscano la sintesi delle chinasi proteiche nucleari cAMP-dipendenti e/o il loro trasferimento nel nucleo e, contemporaneamente, stimolino la sintesi delle chinasi citoplasmatiche. Per converso, si è appurato, in parallelo, che il cAMP ha effetti alquanto limitati sul destino del cortisolo (e metaboliti) legato a proteine nella cellula epatica.

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On the Activity of Cathepsin C in Human Embryonic Kidney Cell Cultures Infected with *Herpesvirus hominis* (*Herpes simplex*)

One of the possible results of the viral infection of susceptible cells in tissue culture are cytopathic effects (CPE), morphological and physiological changes which result from a changed metabolism and an interruption of the stimulation for the cellular replication.

The importance of lysosomal enzymes for the majority of biological processes was obvious soon after the discovery of these subcellular structures[1,2]. The damage of the lysosomes and the release of their enzymes can in certain diseased conditions and after death cause the lysis of the cell.

Already ALLISON and MALLUCCI[3,4] established that release of lysosomal enzymes into the cytoplasm makes an important contribution to cytopathic effects. FINE et al.[5] reported that cells infected with *Herpes simplex* virus undergo different lysosomal and cytopathic changes, which could be correlated with increased accumulation of acid phosphatase and infectious virus in the extracellular fluid. LA PLACA et al.[6] found that acid phosphatase and β-glucuronidase are released from the cells after infection with some toga- and enteroviruses. Lactic dehydrogenase and β-glucuronidase were detected in the tissue culture fluid and in the cellular lysates from cell cultures infected with rabies virus[7]. The activation of lysosomal enzymes was detected from the day 4 of infection on without any indication of CPE. REEVES and CHANG[8] investigated acid phosphatase in the cell infected with vaccinia, fowl plague virus, poliovirus type 2 and adenovirus type 4. The increase of enzyme activity accompanied the first detectable CPE.

It seemed therefore of some interest to test the hypothesis that cathepsins as lysosomal components are an important factor contributing to the destruction of cells infected with viruses. The effect of these hydrolytic enzymes was already investigated in organisms after ionizing irradiation[9,10].

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