EFFECTS OF HORMONES ON CYCLIC AMP RELEASE IN PERFUSED RAT LIVERS

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1. Introduction

The *in vitro* perfusion of rat livers has proven to be a valuable tool for the investigation of the mechanism of action of glucagon, epinephrine and insulin on intact liver cells [1–7]. Their effects on adenylate cyclase and the concomitant changes in cyclic AMP (adenosine 3′,5′-cyclic monophosphate) production play an important role in the sequence of events from the hormone-receptor interaction to the metabolic response of the cell [1, 17]. As it has been shown that cyclic AMP is released by liver cells in response to glucagon and epinephrine [5, 7–10], a detailed study of the kinetics of this response would allow to correlate it more properly with the kinetics of subsequent metabolic events.

Recirculation of relatively small volumes of perfusate through the liver makes it impossible to keep hormone concentrations constant and may therefore blunt or alter hormonal effects by feed-back mechanism. Switching from recirculating to nonrecirculating perfusion just before the addition of hormone alters the concentration of metabolites in the perfusate before they were modulated by the hormone itself.

These difficulties may be overcome by the nonrecirculating perfusion. With this system, the time course of cyclic AMP output by the liver was followed in the presence of hormones influencing adenylate cyclase activity.

2. Materials and methods

Livers of fed, male rats (200–220 g) were perfused in a nonrecirculating system, a modification of the technique by Scholz [11]. It was previously used in other perfusion studies [12, 13]. Bicarbonate buffer containing 119 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, but no albumin or erythrocytes, was pumped through the livers at a rate of approx. 3.5 ml/min/g liver. The perfusate was saturated with O_2/CO_2 (95%/5%) in a temperature regulated disc oxygenator (35° on the liver surface).

Hormone additions were started after 25 min of perfusion and continued to the end of the experiments. Samples of the perfusate leaving the liver were collected for measurement of cyclic AMP at appropriate time intervals.

Cyclic AMP was determined by the competitive protein binding assay of Gilman [14], Walton and Garen [15] modified by Brown [16].

Each assay tube contained: 0.2 ml of 100 mM Tris-HCl buffer containing 16 mM theophyllin and 20 mM $MgCl_2 \cdot 6\,H_2O$; 0.05 ml of [^{3}H]cyclic AMP (50 nCi, specific activity: 20.7 Ci/mmole, The Radiochemical Centre Amersham, Bucks., U.K.); 0.2 ml of perfusate or Krebs–Ringer-bicarbonate buffer; 0.01 ml of cyclic AMP-standard (0–30 pmoles, Boehringer/Mannheim) or 0.01 ml of Krebs–Ringer-bicarbonate buffer; 0.05 ml of binding protein solution.

After an incubation time of 40 min at 4°, 0.5 ml of a charcoal–albumin suspension (5 g of Norit A, Serva, + 0.9 g of human serum albumin + 100 ml of potassium phosphate-buffer, pH 6, 20 mM) were added to each tube and the incubation continued for another 40 min. After centrifugation of the tubes (3000 *g* for 20 min at 4°) a 0.2 ml portion of the supernatant was added to 5 ml of scintillation mixture (Instagel, Packard) and counted for radioactivity in a liquid scintillation counter (Unilux, Nuclear Chicago).

To prepare cyclic AMP-binding protein, fresh rat livers were homogenized in 2 vol of Tris-EDTA buffer (50 mM Tris-HCl, 4 mM EDTA). The homogenate was

centrifuged at 30,000 g for 15 min. 1 ml aliquots of the supernatant were lyophilized and used after 1:4 dilution with Tris-HCl–EDTA buffer for the assay.

Glucagon was a gift from Eli Lilly Company, insulin was kindly supplied by NOVO (Dr. Schlichtkrull), epinephrine was from Noehringer/Mannheim.

3. Results and discussion

A typical standard curve as well as a curve showing the recovery of various amounts of cyclic AMP added to perfusate are presented in fig. 1. 85% to 115% of the added cyclic AMP were recovered.

0.2 to 0.3 pmoles/0.2 ml perfusate of cyclic AMP were the minimal amounts that were measurable in the assay.

In the absence of hormones the release of cyclic AMP by the perfused liver was low (1.15 ± 0.5 nmoles/100 g rat weight/hr, corresponding to 0.35 ± 0.15 pmoles/0.2 ml perfusate, mean ± SEM of 9 perfusions),

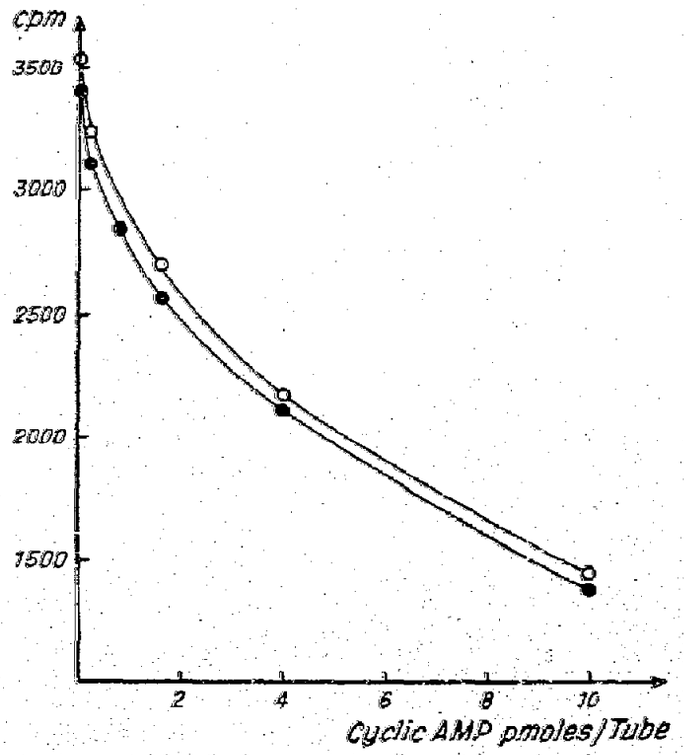

Fig. 1. Typical standard curve (o—o—o) and curve showing the recovery (•—•—•) of various amounts of cyclic AMP (0.2; 0.8; 1.6; 4; 10 pmoles/tube) added to the effluent from a liver perfused with insulin (7.5×10^{-8} M). Assay conditions as described in Materials and methods.

Table 1

Stimulation of cyclic AMP content and cyclic AMP release by 3 different glucagon concentrations.

Glucagon concentrations (M)	Maximal levels of cyclic AMP accumulation, relative to basal level (Exton [1])	Maximal level of cyclic AMP release, relative to basal release (own data)
1×10^{-8}	26×	85×
3×10^{-9}	6×	30×
1.7×10^{-9}	3×	3×

Tissue cyclic AMP levels were taken from [1].

thus confirming the results of another group [7] working with a different perfusion system.

The time course of cyclic AMP output in response to 3 different concentrations of glucagon is shown in fig. 2. Increasing glucagon concentrations resulted in a progressive enhancement of cyclic AMP release by the liver. The dose response relationship was best seen at glucagon concentrations between 1×10^{-9} M and 1×10^{-8} M. In the presence of glucagon concentrations of 1×10^{-9} M or lower the stimulation of cyclic AMP release appeared to be too small to be reliably measured in this system, however glycogenolysis was significantly increased (unpublished). The first rise of cyclic AMP concentration above base-line was usually observed 60 sec after the beginning of glucagon infusion. Infusion of 1×10^{-8} M glucagon resulted in a progressive rise of cyclic AMP output with maximal values of 90–100 nmoles/100 g rat weight/hr after 8 min. This represents an 85-fold increase of basal production rates. During the next 20 min, the nucleotide concentration did not significantly change and then slowly declined (not shown).

Addition of glucagon in a concentration of 3×10^{-9} M to the perfusate led to a transient maximal cyclic AMP release (35 nmoles/100 g rat weight/hr) after 6 min, which represents a 30-fold increase of the basal production rate. After 10 min a steady state was reached at about 20 nmoles/100 g rat weight/hr.

Despite the continuous exposure of the livers to glucagon a secondary decrease of cyclic AMP output was always observed, but it occurred earlier at lower glucagon concentrations.

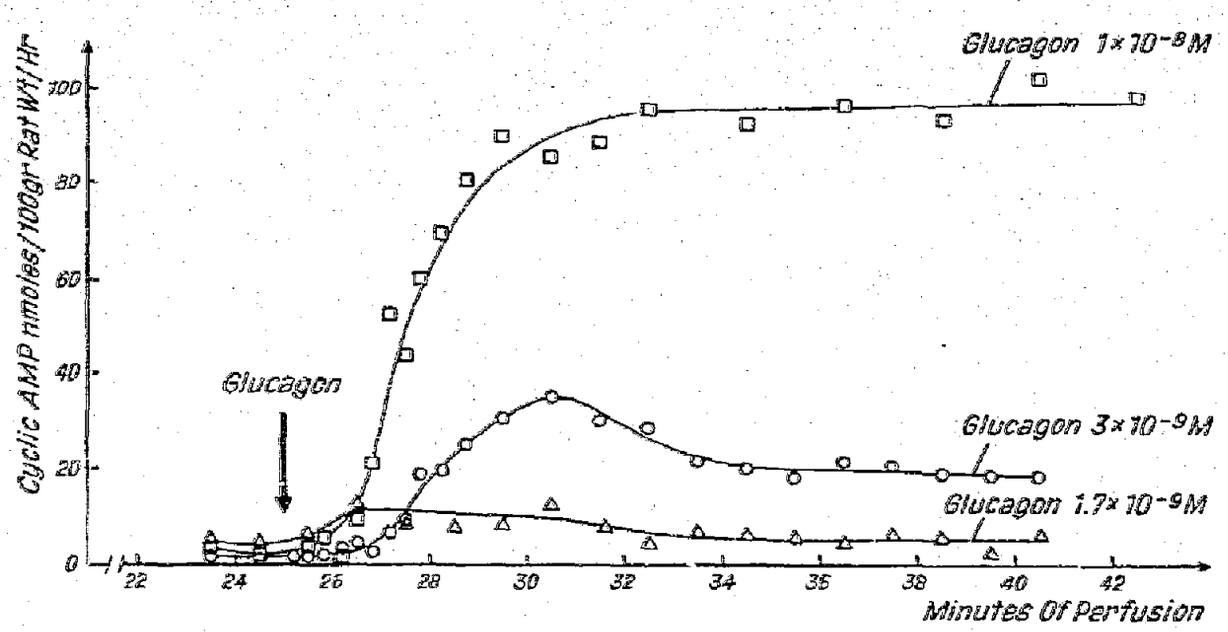

Fig. 2. Time course of cyclic AMP release into the perfusion medium in response to 3 different concentrations of glucagon. Glucagon was infused continuously into the medium entering the liver (final conc.: (△—△—△) 1.7×10^{-9} M; (○—○—○) 3×10^{-9} M; (□—□—□) 1×10^{-8} M). The effluent was collected for the measurement of cyclic AMP at time intervals from 20 to 60 sec. Further experimental details are given in Materials and methods.

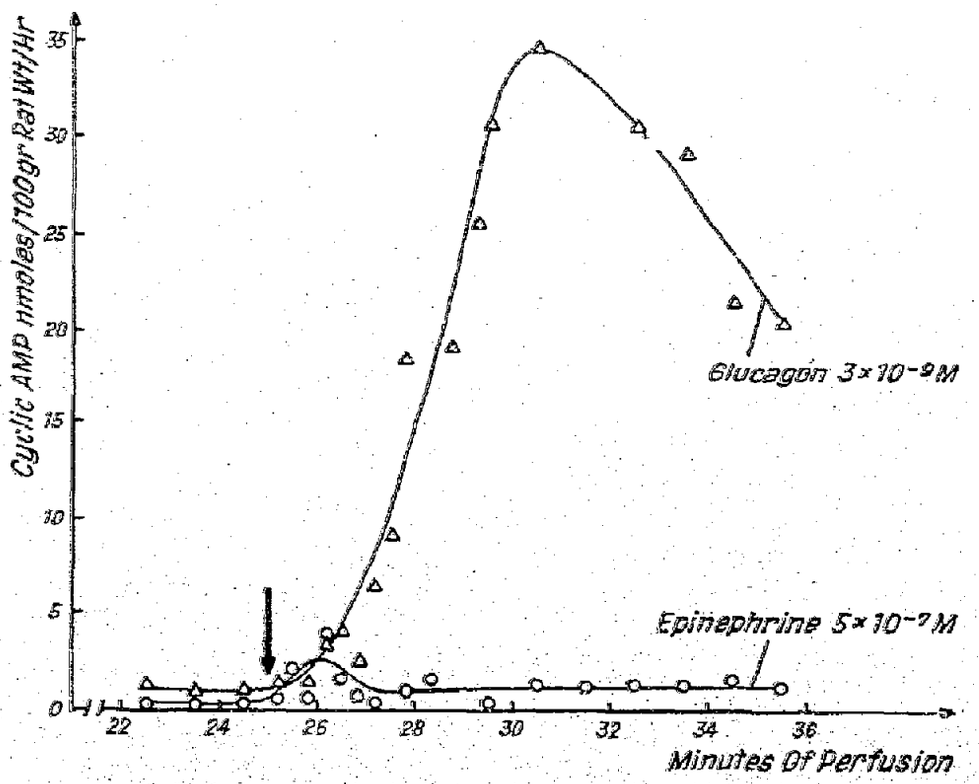

Fig. 3. Comparison of the effects of glucagon (3×10^{-9} M) and epinephrine (5×10^{-7} M) on cyclic AMP release into the perfusion medium. The arrow indicates the start of the hormone infusions. Experimental conditions according to fig. 2 and Materials and methods.

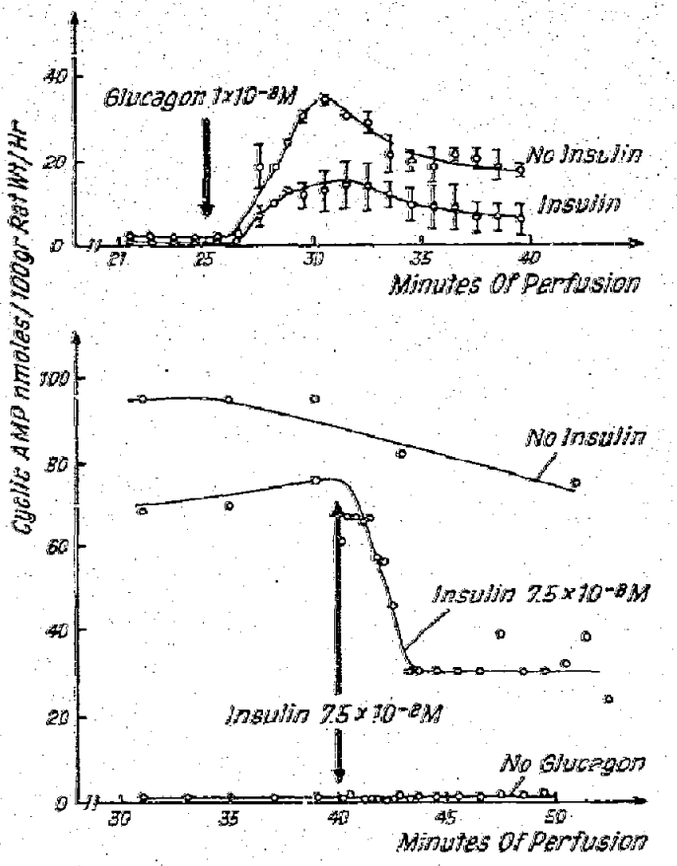

Fig. 4. Interaction of glucagon and insulin in the control of cyclic AMP release by the perfused livers. *Upper panel*: After a 20 min equilibration period insulin infusion (7.5×10^{-8} M) and 10 min later glucagon infusion (3×10^{-9} M) were started. Points are means (± range) of cyclic AMP release in two perfusions. *Lower panel*: After 20 min of perfusion glucagon infusion (1×10^{-8} M) and 20 min later insulin infusion (7.5×10^{-8} M) were started. For further details refer to Materials and methods.

A comparison of the percent increase of cyclic AMP in response to glucagon between the data obtained by Exton et al. [1] for tissue cyclic AMP levels and our results on cyclic AMP release is shown in table 1. In response to increasing doses of glucagon cyclic AMP release increased more dramatically than the tissue cyclic AMP content. This may be due to the fact that in the absence of hormones cyclic AMP release was very low whereas significant amounts of cyclic AMP were still measured in the tissue. This observation supports the suggestion that the cyclic AMP release correlates well with the intracellular concentration of 'free' cyclic AMP, which alone is thought to be metabolically effective (Exton).

As shown in fig. 3 even a large dose of epinephrine (5×10^{-7} M) was much less effective than low doses of glucagon (3×10^{-9} M). There may be a short transient increase of cyclic AMP release at first and a slight gradual rise during the following 10 min of the infusion.

The low rate of cyclic AMP release into the perfusion medium corresponds to the observation of Exton [1], that even high doses of epinephrine cause only a small increase of intracellular cyclic AMP.

Insulin was found to lower the glucagon-induced cyclic AMP release (fig. 4), if it was infused at relatively high concentrations (7.5×10^{-8} M), this is in agreement with the findings of Park et al. [5]. Insulin alone had no effect on basal cyclic AMP release (fig. 4).

It was of particular interest to check whether insulin might change the kinetics of the response to glucagon. When the infusion of insulin was started 10 min prior to that of glucagon the time course appeared to be identical to the one in the absence of insulin. The cyclic AMP output in response to glucagon was reduced by approx. 50% in the presence of insulin.

In conclusion, isolated rat livers perfused in the absence of albumin and erythrocytes were found to respond to hormones as do livers in more complex perfusion systems. The fact that the perfusate is not recirculated through the liver allows to prevent feedback effects of metabolites released into the perfusate, a simple and sensitive assay for cyclic AMP in the perfusate offers the additional possibility to monitor the time course of cyclic AMP release at intervals of 20 sec. This was of interest since the cyclic AMP released into the medium is thought to be in close relation to the intracellular concentration of the "free" or metabolically active nucleotid .

This experimental model appears to be of advantage for further studies on the mechanism of action of hormones on the liver.

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