AN EFFECT OF GLUCAGON ON 3',5'-CYCLIC AMP PHOSPHODIESTERASE ACTIVITY IN ISOLATED RAT HEPATOCYTES

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SUMMARY

Glucagon was found to activate the low Km form of 3',5'-cyclic AMP phosphodiesterase in intact isolated rat hepatocytes while the high Km phosphodiesterase was unaltered. Activation was concentration dependent and occurred at the same concentration required to observe an increase in 3',5'-cyclic AMP levels in the cell. The maximal increase in activity occurred within 5 minutes of incubation with glucagon and was sustained for the 35 minutes assayed.

INTRODUCTION

Insulin has been shown to alter phosphodiesterase activity in liver plasma membranes (1). Preliminary experiments from this laboratory failed to detect any change in phosphodiesterase activity in intact hepatocytes incubated with insulin. However, during these experiments an increase in low Km phosphodiesterase activity was found on incubating the cells with high concentrations of glucagon.

MATERIALS

Collagenase, Batch No. CLS 11 44A185, was purchased from Worthington Biochemicals and Bovine Serum Albumin Powder, Fraction V, (BSA) from Armour Pharmaceuticals. Crystalline Glucagon, Lot No. 258-234B167-1, was a gift from Dr McGuire of Eli Lilly and Co. (Indianapolis, Ind., U.S.A.). [^{3}H] cyclic AMP (30Ci/mmole) was obtained from the Radiochemical Centre (Amersham), while cyclic AMP, adenosine, and Crotalus atrox snake venom were the products of Sigma Chemical Co. Anion exchange resin AG1-X2,200-400 mesh

Cl^- form was purchased from Bio-Rad Laboratories. All other chemicals used were of analytical grade.

METHODS

Hepatocytes were prepared by a modification of the method of Berry and Friend (2). Male Wistar rats (140-200g) were anaesthetized (30mg/kg nembutal i.p.) and 250 units of heparin were injected into the femoral vein. The bile duct was ligated and the liver perfused *in situ* with 50 ml of Ca^{++} free (25mM $NaHCO_3$) modified Hanks buffer pH 7.4 at 37°C (3). The liver was removed and perfused with a further 20 ml of modified Hanks buffer before being connected to the perfusion apparatus (4). The recirculating perfusion medium, which was gassed with 95% O_2/5% CO_2, consisted of 75 ml of modified Hanks buffer containing 0.3% (w/v) collagenase. Perfusion time varied between 19 and 36 min.

Once removed from the perfusion apparatus the liver was placed in a petri dish and covered with Krebs-Ringer bicarbonate buffer pH 7.4 at 37°C. Two spatulas were used to cut the liver capsule and tease the cells from the connective tissue before filtering through nylon gauze. The cells were centrifuged briefly at low speed, washed in 50 ml of Krebs-Ringer bicarbonate buffer at 37°C followed by centrifugation, this repeated 5 times, and the cells finally refiltered. Cell viability was measured with trypan blue and for the experiments described exclusion was greater than 86%.

In all of the experiments cells were suspended in Krebs-Ringer bicarbonate buffer at 37°C and pH 7.4, dispensed into plastic containers, flushed with 95% O_2/5% CO_2, sealed and incubated in a Dubnoff metabolic incubator at 90 oscillations/min for the required time. Protein was estimated by the method of Lowry *et al* (5) using crystalline BSA as standard.

Cyclic AMP Assay

After incubation with glucagon for appropriate times cells were

removed, placed in a boiling water bath for 5 min, then sonicated for 3 min (Dawe Soniprobe setting 4). A $Ba(OH)_2$-$ZnSO_4$ precipitation was carried out at 0°C and the samples assayed for cyclic AMP by the method of Gilman (6).

The validity of the $Ba(OH)_2$-$ZnSO_4$ precipitation procedure was checked by comparing it with the TCA-ether extraction method described by Gilman (6). Since cyclic AMP values were found to be essentially the same in both cases the former method was utilized as the samples could be prepared for assay more quickly.

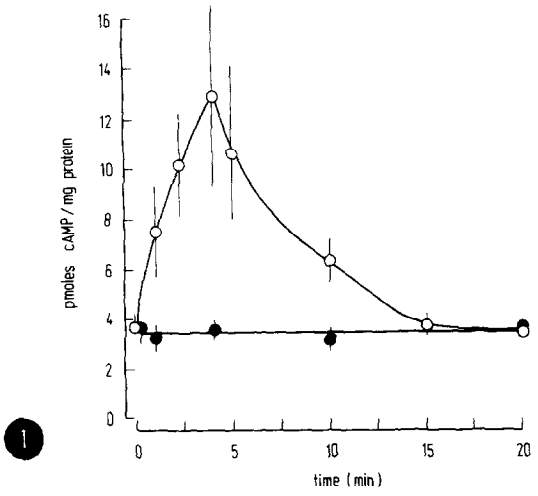
Phosphodiesterase Assay

After incubation in the presence or absence of glucagon the cells were removed, placed on ice and then centrifuged at 1300xg for 6 min at 4°C. The cells were resuspended in 0.25M mannitol-0.025M Tris HCl-1% w/v BSA pH 7.5 before being disrupted by sonication (2 sec/ml, setting 4), diluted 1 in 75 for low Km and 1 in 5 for high Km phosphodiesterase. Phosphodiesterase activity was assayed by a method described previously (7) using cyclic AMP concentrations of 1μM and 250μM for the low and high Km forms respectively and was expressed as pmol adenosine formed/min/mg protein.

RESULTS AND DISCUSSION

The Effect of Glucagon on Hepatocyte Cyclic AMP

Hepatocytes prepared from 24 hr starved rats were preincubated for 5 min prior to the addition of glucagon (final concentration 2.7μM). After addition samples containing $10\text{-}20\times10^6$ cells were removed at intervals for cyclic AMP assay. Fig. 1 shows that exposure of isolated hepatocytes to glucagon produced a maximal increase in cyclic AMP of 3.6 fold 4 min after the addition of glucagon and cyclic AMP levels subsequently returned to basal after 15 min. Although the maximal increase in cyclic AMP levels observed here is low when compared with other communications



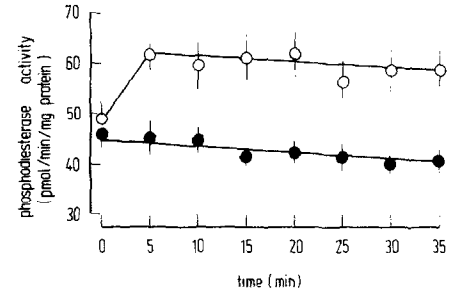


Fig. 1. Time course of cAMP levels in isolated hepatocytes in the presence O and absence ● of 2.7μM glucagon. Each point is the mean ± S.E.M. of 4 experiments.

Fig. 2. The effect of glucagon concentration on the low km form of phosphodiesterase in hepatocytes. Except where indicated in parenthesis each point is the mean ± S.E.M. of 5 experiments.

(8, 9, 10),it has been found in perfused rat livers that a 4 fold elevation of cyclic AMP stimulates gluconeogenesis maximally (11) and in rat epididymal fat pads (12) a 2 fold increase in cyclic AMP is all that is required to stimulate lipolysis maximally. The variation in response to glucagon (8, 9, 10) may be due to the exposure of the liver to different batches of crude collagenase during the isolation procedure. This could affect the membrane receptors which would influence the sensitivity of the cell to glucagon.

The Effect of Glucagon Concentration on Phosphodiesterase

Samples containing approximately $3x10^6$ cells were incubated for 9 min with glucagon concentrations ranging from O to 27μM and the phosphodiesterase measured as described above.

The increase in phosphodiesterase activity produced by incubation of hepatocytes with glucagon was seen only under assay

Table I

Effect of Glucagon Concentration on High Km Phosphodiesterase Activity in Hepatocytes

Glucagon Molarity	Phosphodiesterase Activity (pmoles/min/mg protein)
0	2520 ± 500
2.7×10^{-12}	2660 ± 440
2.7×10^{-11}	2700 ± 340
2.7×10^{-10}	2680 ± 450
2.7×10^{-9}	2600 ± 380
2.7×10^{-8}	2400 ± 380
2.7×10^{-7}	2540 ± 340
2.7×10^{-6}	2640 ± 400
2.7×10^{-5}	2440 ± 510

Results are the means ± S.E.M. of 5 experiments except where indicated in parenthesis.

conditions which favoured the enzyme with a relatively high affinity for cyclic AMP (Fig. 2). The maximal stimulation of this form of phosphodiesterase occurred at the same concentration of glucagon (2.7μM) used to obtain a measurable increase in cyclic AMP levels in the cells (Fig. 1). However, there was no significant change in high Km phosphodiesterase for any of the experiments described (Table I).

The low Km form of the enzyme is thought to be associated with liver plasma membranes (13) and the plasma membrane of fat cells (14). As yet, however, we have not attempted to isolate membranes from hepatocytes to confirm this.

This effect on phosphodiesterase appears to be a property of intact hepatocytes since no stimulation of phosphodiesterase could

Table II

Effect of Glucagon Concentration on Cell Homogenates

Glucagon Molarity	Phosphodiesterase Activity (pmol/min/mg protein)	
	Low Km	High Km
0	43	2660
2.7×10^{-12}	39	2560
2.7×10^{-11}	45	2550
2.7×10^{-10}	44	2590
2.7×10^{-9}	45	2620
2.7×10^{-8}	43	2590
2.7×10^{-7}	41	2610
2.7×10^{-6}	36	2600
2.7×10^{-5}	36	2490

The procedure used was essentially that described for the effect of glucagon concentration on phosphodiesterase activity with the exception that glucagon was added immediately after disruption of the cells. Results are the means of 2 experiments.

be observed when glucagon was added after the disruption of the cells (Table II).

Phosphodiesterase Activity as a Function of Time

A suspension containing approximately 3×10^6 cells/ml was incubated with 2.7μM glucagon and aliquots removed at 5 min intervals for phosphodiesterase assay. Activation was compared with a similar suspension incubated without glucagon. Fig. 3 shows that the maximal stimulation of the low Km form was attained within 5 min of the addition of glucagon. Once stimulated the activity remained unchanged for at least 35 min. These results differ from those observed in intact fat cells by Pawlson *et al* (15) and by Zinman and Hollenberg (16) where a sustained activation of low Km

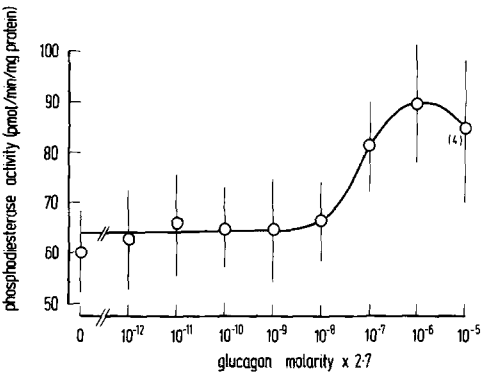


Fig. 3. Low km phosphodiesterase as a function of time in isolated hepatocytes incubated in the presence 0 and absence ● of 2.7μM glucagon. Each point is the mean ± S.E.M. of 5 experiments.

phosphodiesterase was produced only by insulin. However, with all the cyclic AMP-stimulating hormones studied maximal activity was reached within 5 min (15, 16). In contrast, House et al (1) found that stimulation of phosphodiesterase from rat liver plasma membran occurred less rapidly but continued to rise for at least 20 min.

Although no decline in phosphodiesterase activity could be observed within 35 min the results obtained here would appear to follow more closely those found in intact fat cells (15, 16) and agree with the hypothesis put forward for fat cells that there is a component of phosphodiesterase which can be modified by its substrate concentration.

ACKNOWLEDGEMENT

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