Variations in the antagonistic effects of insulin and glucagon on glycogen metabolism in cultured foetal hepatocytes

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The antagonistic effects of insulin and glucagon on glycogen formation and mobilization were studied in cultured 18-day foetal rat hepatocytes with regard to different modes of exposure. Hormone combinations were achieved with a constant dose of 10 nM-insulin (maximal for the glycogenic effect of this hormone) and increasing doses of glucagon [from 0.03 to 10 nM; concn. causing half-maximal response (ED_{50}) = 0.3 nM]. When insulin and glucagon were added simultaneously, increasing glucagon concentrations progressively depressed the glycogenic effect of insulin and 0.3 nM-glucagon antagonized the insulin effect completely. The maximal glycogenolytic effect of glucagon was observed at concentrations > 1 nM. When the two hormones were introduced successively, with an interval of 4 h between additions, the effect of the second hormone was always fully expressed between 4 and 8 h, at which time the effect of the first hormone had ceased; the dominance of glucagon over insulin was also lost, due to cell desensitization to glucagon. Both continuous or intermittent (10 min on/10 min off periods) exposure to insulin and/or glucagon gave similar antagonistic effects, while in cells exposed to insulin plus glucagon alternating with exposure to insulin or glucagon alone, the glycogenic effect of insulin was less or more antagonized respectively by glucagon. Whatever the situation, the results obtained could not be related to antagonism by a glucagon-induced rise in either cyclic AMP levels (ED_{50} = 3 nM) or cell-surface hormone binding. Thus, depending on the hormonal state and the mode of hormone administration, regulation of glycogenesis in cultured foetal hepatocytes appears to be different from that predicted by the insulin/glucagon molar ratio, which is strikingly altered in the perinatal period.

INTRODUCTION

Insulin and glucagon play major roles in hepatic carbohydrate metabolism via antagonistic actions. The insulin/glucagon molar ratio, high during feeding periods followed by liver glycogen storage, and low during the deprivation periods, has been found to regulate hepatic glucose homeostasis in the adult (for reviews, see Unger, 1983; Cherrington *et al.*, 1987). Furthermore, the insulin/glucagon molar ratio in the portal vein is related to the pulsatility of insulin and glucagon release by the pancreas (for reviews, see Lefèvre *et al.*, 1987; Weigle, 1987). Glucose, insulin and glucagon regulate both hepatic glucose production and net hepatic glucose uptake in the conscious dog (Cherrington *et al.*, 1987). The antagonistic effects of insulin and glucagon on hepatic glucose production have been particularly studied in the perfused adult liver (Mackrell & Sokal, 1969; Exton *et al.*, 1972; El-Refai & Bergman, 1979; Komjati *et al.*, 1986). The antagonism can be explained, at least in part by receptor-mediated opposite changes in cyclic AMP concentrations, which in turn regulate the activities of enzymes of glycogen metabolism (for reviews, see Stalmans, 1983; Houslay, 1986; Exton, 1987; van de Werve & Jeanrenaud, 1987). In the foetal rat liver, glucose production is absent because of the lack of glucose-6-phosphatase activity, whereas glycogen synthesis is very active in late gestation (Dawkins, 1963). The insulin/glucagon molar ratio is high in foetal rat plasma and decreases dramatically at birth (Girard *et al.*, 1974). The apparent agreement between changes in this ratio and the prompt mobilization at birth of glycogen stored during the perinatal period has remained debatable (Girard *et al.*, 1973; Snell & Walker, 1978; Moncany & Plas, 1980; review in Ktorza *et al.*, 1985).

When 18-day foetal-rat hepatocytes are grown in the presence of cortisol, they synthesize and accumulate glycogen (Plas *et al.*, 1973). In addition, a striking stimulation of glycogen formation occurs in the presence of insulin (Plas *et al.*, 1979; DeSante *et al.*,

1984), whereas glucagon addition produces a considerable mobilization of glycogen (Plas & Nunez, 1975). Foetal hepatocytes show time-dependent responses to insulin and to glucagon, with desensitization occurring on previous exposure to the hormone (Plas & Nunez, 1975; Plas *et al.*, 1979; Moncany & Plas, 1980). The dual regulation of glycogen metabolism was taken into account to investigate the effect of modification of the insulin/glucagon molar ratio with regard to hormonal presence (continuous, intermittent or alternative). This study demonstrates that the insulin/glucagon molar ratio is an important factor for the regulation of glycogen metabolism, which is largely dependent on the mode of hormone exposure and the hormonal impregnation state.

MATERIALS AND METHODS

Materials

Insulin and glucagon were purchased from Novo Laboratories. D-[U-¹⁴C]Glucose was from New England Nuclear. The sources of other materials have been specified previously (Plas & Nunez, 1975).

Culture procedure

Primary cultures of hepatocytes were obtained from 18-day rat foetuses (Sprague–Dawley rats) as described previously (Plas *et al.*, 1973). After mild trypsin treatment, the isolated cells were plated on a collagen substratum to which only the hepatocytes adhered, and after 6 h the non-adhering haematopoietic cells were removed. At this step, and after 24 and 48 h, the culture medium (1 ml/well) was replaced. It consisted of NCTC 109 medium (Evans *et al.*, 1964) containing glucose at a concentration of 5.5 mM and supplemented with 10% (w/v) foetal calf serum and 10 μ M-cortisol. All experiments were performed after 2 days of culture, using a hormone combination of 10 nM-insulin (a

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maximal concentration for the glycogenic effect) and increasing concentrations of glucagon (from 0.03 to 10 nM). At this time, the glucose concentration in the medium was close to 4 mM, which corresponds to the glycaemia of the rat foetus at the end of gestation.

Glycogen studies

Glycogen content and glycogen labelling were measured in the presence of [^{14}C]glucose as described previously (Plas *et al.*, 1973). Two sets of labelling experiments were carried out. (1) In continuous-labelling experiments, [^{14}C]glucose was added to the medium at the start of the culture to give a final specific radioactivity of 0.25 $\mu\text{Ci}/\text{mg}$, which was maintained throughout the culture period. On day 2, insulin and/or glucagon was added and glycogen was extracted after various incubation periods. Parallel measurements of the radioactivity present in glycogen and of glycogen content allowed calculation of the specific radioactivity of the glucose residues in the stored glycogen. The contribution of medium glucose to glycogen formation is expressed by the ratio a/b , where a is the specific radioactivity of glucose units in glycogen and b is the specific radioactivity of glucose in the medium. (2) In short-labelling experiments, [^{14}C]glucose (1 $\mu\text{Ci}/\text{mg}$) was added at day 2 of culture together with insulin and/or glucagon. The radioactivity present in glycogen was determined after various incubation periods under conditions which are specified for each experiment.

Cyclic AMP studies

The cellular content of cyclic AMP was measured as already described (Moncany & Plas, 1980). After incubation in the presence of hormones, the culture medium was discarded, and the cells were rapidly frozen in 50% acetic acid on solid CO_2 . After extraction of the cellular material, the acetic acid was evaporated at 80 $^{\circ}\text{C}$ and the residue was dissolved in 50 mM-acetate buffer, pH 6.2. The concentration of cyclic AMP was estimated by the radioimmunoassay procedure of Steiner *et al.* (1972).

Cell-surface insulin and glucagon binding studies

Cell-surface insulin and glucagon binding after a transitory exposure to native hormone was determined by the method previously described (Soubigou *et al.*, 1986), with the following modifications. Cultured foetal hepatocytes were first exposed to 10 nM-insulin and/or 3 or 10 nM-glucagon in the culture medium at 37 $^{\circ}\text{C}$ for 10 min or 4 h; cell-surface binding was then measured at 4 $^{\circ}\text{C}$ as follows. Cultures were washed once with binding medium containing 10 nM- ^{125}I -insulin or 10 nM- ^{125}I -glucagon and incubated for 4 h in the same medium. Dissociation of cell-surface-bound insulin and glucagon occurred effectively during the conditions of this exchange binding assay, since identical results were obtained with a second protocol when cell-surface-bound hormone was first removed by acid treatment before incubation in binding medium. Corrections were made for the non-specific association of ^{125}I -insulin or ^{125}I -glucagon with cells by performing parallel incubations in the presence of 10 μM -hormone.

Presentation of results

Each protocol involved at least three independent experiments performed on different cell preparations. Data are presented as means $\pm$ S.E.M. for the numbers of independent experiments indicated (n). When a representative experiment is presented, each symbol on the graphs corresponds to the mean of triplicate culture measurements. The results are expressed per mg of protein. The cell population of a culture well was of the order of 0.6×10^6 hepatocytes, which corresponds to 230 μg of protein

and to 1.60 mg wet wt. of liver. In order to express insulin or glucagon response, a 'stimulation or inhibition index' was used, defined as the following ratio: nmol of glucose in glycogen/mg of cell protein in treated cultures divided by nmol of glucose in glycogen/mg of cell protein in control cultures.

RESULTS

Antagonistic effects of insulin and glucagon on glycogenesis when hormones were added separately, simultaneously and successively

The dose-response relationship of the antagonism of the hormone effects was first determined by using 4 h labelling experiments, in which [^{14}C]glucose was added together with various doses of insulin or glucagon, or various doses of glucagon plus 10 nM-insulin. The maximal glycogenic response (stimulation index 3.9) was obtained with 10 nM-insulin and the half-maximal response was obtained with a dose close to 0.3 nM (ED_{50}) (Fig. 1). When glucagon was considered, a maximal inhibition of glycogen labelling (inhibition index 0.22) was obtained at concentrations greater than 3 nM-glucagon, with the half-maximal response at 0.3 nM-glucagon. When a dose of 10 nM-insulin was added together with increasing doses of glucagon (from 0.03 to 10 nM), the glycogenic effect of insulin progressively disappeared, and was completely suppressed by a dose of glucagon as low as 0.4 nM. In the presence of 3–10 nM-glucagon, a weak inhibitory effect of 10 nM-insulin was observed on the glucagon-induced glycogenolytic response.

The antagonism between the effects of insulin and glucagon was also studied by parallel measurement of glycogen content and radioactivity present in glycogen in continuous-labelling experiments. At the time of hormone addition (day 2 of culture), the hepatocytes contained appreciable amounts of stored glycogen (165 μg of glycogen/mg of protein) which had been synthesized *de novo* in culture (Plas *et al.*, 1973). When the two hormones were added separately at a maximal 10 nM concentration, insulin increased by 50% the stored glycogen, whereas glucagon produced a mobilization which represented, after 4 h,

Fig. 1. Dose-dependent antagonistic effects of insulin and glucagon on [^{14}C]glucose incorporation into glycogen

At day 2, various doses of insulin and/or glucagon were introduced into the medium together with [^{14}C]glucose (1 $\mu\text{Ci}/\text{mg}$), and glycogen labelling was measured 4 h later. Cultures incubated with various doses of insulin alone (○), glucagon alone (□) or glucagon in the presence of 10 nM-insulin (●) are represented.

Fig. 2. Time-dependence of the antagonistic effects of insulin and glucagon on glycogen content

At day 2, cultures first received 10 nM-insulin, 10 nM-glucagon, 10 nM-insulin plus 10 nM-glucagon (or 0.3 nM-glucagon), or hormone solvent alone. After 4 h, the first three sets of cultures were subsequently exposed to 10 nM-insulin, 10 nM-glucagon or hormone solvent, and incubated for a further 4 h. Glycogen content was determined during 8 h. Cultures incubated after the first hormone addition during 4 h with 10 nM-insulin (○), 10 nM-glucagon (□), 10 nM-insulin plus 10 nM-glucagon (●) or 0.3 nM-glucagon (Δ), or hormone solvent (X), and parallel cultures incubated for 8 h which had received after 4 h 10 nM-insulin (○), 10 nM-glucagon (□) or hormone solvent (X) as a second hormone addition, are represented.

60% of the initial glycogen content (Fig. 2). Simultaneous addition of insulin and glucagon at 10 nM produced a time-dependent glycogen mobilization similar to that obtained with 10 nM-glucagon alone, in agreement with the results shown in Fig. 1. In the presence of a dose of glucagon close to its ED_{50} for the glycogenolytic response, insulin was able to counteract the glycogenolytic effect of glucagon (Fig. 2), which was nevertheless clearly expressed during the first 15–30 min (results not shown). In a parallel set of cultures, the first dose of 10 nM-insulin and/or 10 nM-glucagon was followed 4 h later by the addition of 10 nM-

glucagon or 10 nM-insulin respectively. The glycogen content was measured 4 h later. After preincubation with 10 nM-insulin, glucagon produced a maximal mobilization of glycogen, at a time when no response to a further dose of insulin could be obtained (Fig. 2). Similarly, after preincubation with 10 nM-glucagon, the effect of 10 nM-insulin was maximally expressed once the glycogenolytic response to glucagon had ceased and could not be initiated again by a fresh dose of glucagon. After preincubation in the presence of 10 nM-insulin together with 10 nM-glucagon, a further dose of glucagon or of insulin was totally ineffective.

The kinetic variations observed in glycogen labelling during continuous-labelling experiments were similar to those obtained for glycogen content (results not shown). From the ratios of the specific radioactivity of glucose residues in glycogen to that of glucose in the medium, it was possible to determine the relative contribution of glucose to glycogen formation. At the time of hormone addition, the relative contribution of glucose to glycogen formation was approx. 63%. This value varied in opposite directions after a 4 h incubation in the presence of 10 nM-insulin (increased by 20%) or 10 nM-glucagon (decreased by 23%) (Table 1). When the antagonism between the effects of insulin and glucagon was total, i.e. 0.3 nM-glucagon plus 10 nM-insulin, the contribution of glucose was similar to that obtained in untreated cells (approx. 60%). When an initial 4 h incubation with 10 nM-insulin (or 10 nM-glucagon) was followed by the addition of 10 nM-glucagon (or 10 nM-insulin), the contribution of glucose to glycogen after 8 h was decreased by 28% (or increased by 24%) as compared with the values obtained after 4 h. Thus the successive addition of the antagonistic hormone produced the same effect on the contribution of glucose to glycogen formation as that observed after separate addition of hormones.

Antagonistic effects of insulin and glucagon on glycogenesis in cells exposed to hormones intermittently or alternately

Hepatocytes were first exposed to various doses of glucagon (0.03–3 nM), either (1) continuously, or (2) intermittently by using four successive short exposures to glucagon for 10 min

Table 1. Contribution of glucose to glycogen synthesized in the presence of insulin and/or glucagon in cultured foetal hepatocytes

In the continuous-labelling experiments, [14 C]glucose (0.25 μ Ci/mg) was present in the medium throughout the culture period. Insulin (10 nM), 10 nM-insulin and/or increasing concentrations of glucagon, or hormone solvent alone, were added at day 2 of culture. Glycogen content and radioactivity present in glycogen were determined at the various times indicated; the ratio a/b expresses the contribution of the glucose medium to glycogen formation. The results shown are representative of at least three independent experiments performed with different cell preparations.

Addition		Duration of incubations (h)	Glycogen (μ g/mg of protein)	Specific radioactivity of glucose (μ Ci/mg)		a/b	
$t = 0$	$t = 4$ h			In glycogen (a)	In medium (b)	4 h	8 h
None	—	0	165	0.156	0.25	—	—
None	—	4	171	0.157	0.25	0.63	—
10 nM-Insulin	—	4	229	0.190	0.25	0.76	—
10 nM-Glucagon	—	4	63	0.123	0.25	0.49	—
0.3 nM-Glucagon + 10 nM-insulin	—	4	138	0.158	0.25	0.63	—
10 nM-Glucagon + 10 nM-insulin	—	4	73	0.130	0.25	0.52	—
10 nM-Insulin	10 nM-Glucagon	8	105	0.138	0.25	—	0.55
10 nM-Glucagon	10 nM-Insulin	8	57	0.161	0.25	—	0.64
10 nM-Glucagon + 10 nM-insulin	None	8	56	0.148	0.25	—	0.59
10 nM-Glucagon + 10 nM-insulin	10 nM-Insulin	8	50	0.155	0.25	—	0.62
10 nM-Glucagon + 10 nM-insulin	10 nM-Glucagon	8	73	0.151	0.25	—	0.60

Fig. 3. Dependence of the antagonistic effects of insulin and glucagon on [^{14}C]glucose incorporation into glycogen on the mode of exposure

At day 2, the medium was replaced by fresh NCTC 109 medium supplemented with increasing doses of glucagon (0.03–3 nM) and/or 10 nM-insulin or hormone solvent, together with [^{14}C]glucose (1 $\mu\text{Ci}/\text{mg}$ of glucose). Four successive exposures to glucagon and/or insulin at 10 min intervals, separated by incubations in the presence either of hormone solvent or of the specified hormone, were used. Cultures were then incubated for up to 70 min, [^{14}C]glucose being maintained in the medium, which was replaced every 10 min. The radioactivity present in glycogen is shown for cultures exposed: (a) intermittently ($\square$) or continuously ($\blacksquare$) to increasing doses of glucagon; (b) intermittently ($\circ$) or continuously ($\bullet$) to increasing doses of glucagon plus 10 nM-insulin; and (c) to increasing doses of glucagon plus 10 nM-insulin, alternating with exposure to the specified hormone alone, which is 10 nM-insulin ($\triangle$) or the corresponding dose of glucagon ($\blacktriangle$).

Fig. 4. Effect of insulin on the glucagon-induced rise in cyclic AMP levels

At day 2, various doses of insulin and/or glucagon were introduced into the medium, and the cyclic AMP content of the cells was determined 4 min after hormone addition. Cultures incubated with insulin alone ($\circ$), or with various doses of glucagon either alone ($\square$) or in the presence of 10 nM-insulin ($\bullet$), are represented. The broken line represents the dose-dependent glycogenolytic response to glucagon, expressed as a percentage of the maximal effect.

each separated by 10 min incubations in the absence of the hormone. Both continuous and intermittent (10 min on/10 min off) exposure to increasing doses of glucagon elicited a similar progressive decrease in [^{14}C]glucose incorporation into glycogen (Fig. 3a). Also, the addition of 10 nM-insulin using the intermittent mode (no glucagon) gave a stimulation of the incorporation of [^{14}C]glucose into glycogen of the same extent as during continuous insulin exposure (Fig. 3b). In three independent experiments, the inhibition index with 0.3 nM-glucagon was 0.65 ± 0.09 versus 0.74 ± 0.11 ($n = 4$, $P > 0.1$) and the stimulation index with 10 nM-insulin was 1.85 ± 0.21 versus 1.79 ± 0.18 ($n = 4$, $P > 0.5$), for continuous and intermittent exposures respectively. The amount of insulin or glucagon remaining in the medium during the 10 min intervals following

Table 2. Variations in the stimulation of cyclic AMP levels in hepatocytes pre-exposed to insulin and/or glucagon

At day 2, cultures received 10 nM-insulin, 10 nM-glucagon, 10 nM-insulin plus 10 nM-glucagon, or hormone solvent alone. After 4 h, each set of cultures was subsequently exposed to 10 nM-insulin, 10 nM-glucagon or hormone solvent. Cyclic AMP levels were determined 4 min after each addition. Results are represented as means $\pm$ S.E.M. for three experiments performed with different cell preparations.

Addition at time zero	Addition at 4 h	Cyclic AMP (pmol/mg of protein)
10 nM-Insulin	10 nM-Insulin	14 ± 1.7
10 nM-Insulin	10 nM-Glucagon	45 ± 7.9
10 nM-Insulin	Solvent	15 ± 1.2
10 nM-Glucagon	10 nM-Insulin	23 ± 2.2
10 nM-Glucagon	10 nM-Glucagon	24 ± 2.7
10 nM-Glucagon	Solvent	23 ± 2.4
10 nM-Insulin + 10 nM-glucagon	10 nM-Insulin	22 ± 2.3
10 nM-Insulin + 10 nM-glucagon	10 nM-Glucagon	24 ± 2.4
10 nM-Insulin + 10 nM-glucagon	Solvent	23 ± 2.8
Solvent	10 nM-Insulin	15 ± 1.9
Solvent	10 nM-Glucagon	46 ± 7.3
Solvent	Solvent	16 ± 1.4

removal of the hormone was negligible, since it represented about 4% of the concentration of the hormone added when determined by using iodinated hormone (results not shown). Thus, with insulin or increasing doses of glucagon using the intermittent mode, a fully expressed glycogenic or glycogenolytic response was obtained, although the total amount of the hormone added was only 57% of that present during continuous exposure. Cells were then exposed continuously or intermittently to increasing doses of glucagon together with insulin maintained at

Table 3. Cell-surface binding of ^{125}I -insulin or ^{125}I -glucagon after exposure to native hormones

Foetal hepatocytes were first incubated in the presence of 10 nM-insulin, 10 nM-glucagon, 10 nM-insulin plus 10 nM-glucagon or hormone solvent alone at 37 °C for 10 min or 4 h. Then, cell-surface hormone binding was measured after incubation for 4 h at 4 °C in binding medium in the presence of 10 nM- ^{125}I -insulin or 10 nM- ^{125}I -glucagon by using the exchange binding assay as described in the Materials and methods section. Results are represented as means $\pm$ S.E.M. for three experiments performed with different cell preparations.

Addition at time zero	Cell-surface-specific hormone binding (fmol/mg of protein)			
	^{125}I -insulin		^{125}I -Glucagon	
	10 min	4 h	10 min	4 h
10 nM-Insulin	376 $\pm$ 38	359 $\pm$ 35	465 $\pm$ 36	470 $\pm$ 31
10 nM-Glucagon	615 $\pm$ 43	630 $\pm$ 42	330 $\pm$ 25	328 $\pm$ 18
10 nM-Glucagon + 10 nM-insulin	380 $\pm$ 36	377 $\pm$ 37	339 $\pm$ 24	330 $\pm$ 17
Solvent	626 $\pm$ 41	617 $\pm$ 45	470 $\pm$ 36	463 $\pm$ 27

the same 10 nM maximal dose. With the continuous as well as the intermittent mode, increasing glucagon concentrations inhibited progressively the glycogenic effect of insulin, which was totally antagonized at a concentration of 1 nM-glucagon (Fig. 3b), a pattern similar to that shown in Fig. 1.

Parallel experiments were performed to compare the antagonistic effects of glucagon and insulin when the hormones were added alternately to the incubations. For this purpose, cells were exposed alternately to increasing doses of glucagon plus 10 nM-insulin and to one hormone alone, i.e. glucagon at the corresponding dose or insulin at 10 nM (i.e. one hormone is continuously present and the other is intermittently present). When the hormone continuously present was the corresponding dose of glucagon, the glycogenic response to insulin was antagonized by glucagon to a greater extent than when cells were exposed intermittently to various doses of glucagon plus 10 nM-insulin (inhibition index 0.48 ± 0.10 versus 0.68 ± 0.09 , $n = 4$, $P < 0.001$, with 0.3 nM-glucagon) (Fig. 3c). When the hormone continuously present was 10 nM-insulin, the glycogenic effect of insulin was less antagonized by glucagon than in the intermittent mode, whatever the concentration of glucagon considered (inhibition index 0.84 ± 0.11 versus 0.68 ± 0.09 , $n = 4$, $P < 0.01$, with 0.3 nM-glucagon). Thus, as compared with intermittent (as well as continuous) exposure to increasing doses of glucagon plus 10 nM-insulin, the continuous presence of glucagon, or insulin, during periodic exposure of the other hormone revealed respectively a clear accentuation or attenuation of the progressive glycogenolytic effect of glucagon.

Effect of insulin on the glucagon-induced rise in cyclic AMP levels

As glucagon is known to produce rapid cyclic AMP accumulation in foetal hepatocytes (Plas & Nunez, 1975), it was interesting to study whether insulin influenced this process. The intracellular cyclic AMP content was determined 4 min after hormone addition, a time corresponding to the transient peak for the glucagon-induced rise in intracellular cyclic AMP levels (Moncany & Plas, 1980). The extent of the increase depended on the glucagon concentration ($\text{ED}_{50} = 3$ nM), a dose-response relationship which was not modified by the simultaneous presence of 10 nM-insulin, the latter also having no effect when added alone (Fig. 4). Variations in the cyclic AMP levels were also investigated in foetal hepatocytes previously incubated for 4 h under conditions similar to those described in the legend to Fig. 2. In insulin-treated cells, the addition of 10 nM-glucagon produced a rise in cyclic AMP levels similar to that obtained in untreated cells (Table 2). Cyclic AMP levels could not be modified

by a second addition of 10 nM-glucagon, independent of the simultaneous presence of 10 nM-insulin during the first 4 h. The second insulin addition, like the first, had no effect whether glucagon was present or not.

Effects of exposure to insulin and glucagon on cell-surface binding of the antagonistic hormone

Cell-surface binding of ^{125}I -insulin or ^{125}I -glucagon was studied in foetal hepatocytes preincubated with the same or an antagonistic native hormone at 37 °C for 10 min or 4 h. After a 10 min or a 4 h preincubation period in the presence of the unlabelled insulin or glucagon, cell-surface binding of ^{125}I -insulin or ^{125}I -glucagon respectively was decreased compared with that obtained in the absence of unlabelled hormone (by 40 % and 33 % respectively) (Table 3). Cell-surface binding of ^{125}I -insulin was not modified by the presence of glucagon, insulin being present or not during the 10 min or 4 h preincubation period, in particular under conditions when the glycogenic effect of insulin was fully antagonized by glucagon. Similar results were obtained when cell-surface binding of ^{125}I -glucagon, in the presence of insulin, was considered.

DISCUSSION

The antagonistic effects of insulin and glucagon were studied in cultured foetal rat hepatocytes, a culture system in which an acute stimulation by insulin of glycogen synthesis has been demonstrated (Plas & Nunez, 1976; Plas *et al.*, 1979; DeSante *et al.*, 1984). The antagonism between insulin and glucagon has been studied in perfused adult liver with regard to net hepatic glucose production (Mackrell & Sokal, 1969; Exton *et al.*, 1972; El-Refai & Bergman, 1979; Komjati *et al.*, 1986), a pathway not present in cultured foetal hepatocytes (Menuelle *et al.*, 1988). Insulin is thought to have no effect by itself in the adult liver, where it essentially counteracts the effects of glucagon and α_1 -adrenergic agents at the level of hepatic glucose production (Katz & McGarry, 1984; Cherrington *et al.*, 1987). Besides, some stimulatory effect of insulin, used as the sole hormone, on net glycogen synthesis is expressed in primary cultures of adult hepatocytes (Schudt, 1979). In the present study, a variety of time-dependent interactions between the two hormones was explored, the hormone combination being achieved with a constant dose of 10 nM-insulin (maximal for the insulin glycogenic effect) and increasing doses of glucagon (0.03–10 nM), with glucose maintained at 4 mM.

When the two hormones were added simultaneously, increasing glucagon concentrations progressively depressed the

glycogenic effect of insulin, the antagonism being cancelled with 0.3 nM-glucagon. The glycogenolytic effect of glucagon was observed at higher concentrations. For an insulin/glucagon molar ratio equal to or less than 10, glucagon in the presence of insulin appeared as the dominant hormone. This could be related to the delayed response to insulin (about 20 min) as compared with the immediate one for glucagon, and to the differences existing between the early rates of hormone-induced degradation and synthesis of glycogen (Plas & Nunez, 1975; Plas *et al.*, 1979). In the adult perfused liver, insulin can substantially diminish the stimulatory effect of submaximal concentrations of glucagon on hepatic glucose production (Mackrell & Sokal, 1969; Exton *et al.*, 1972). This has been associated with the ability of insulin to rapidly decrease cyclic AMP levels raised by glucagon, by the stimulation of a phosphodiesterase that degrades cyclic AMP (Blackmore *et al.*, 1979; Heyworth *et al.*, 1983). In cultured foetal hepatocytes, a glucagon-induced rise in cyclic AMP levels had an ED₅₀ ten times higher than that for the glycogenolytic response, insulin being unable to modify cyclic AMP levels. It must be noted that it was not possible to study the effect of 10 nM-insulin in modifying glycogenolysis induced by low concentrations of glucagon, as in this case no significant rise in cyclic AMP levels was observed. The large disparity in the sensitivities to glucagon between glycogenolysis and cyclic AMP accumulation suggests that, in the foetal system, a cyclic AMP-independent mechanism could be involved, as has been suggested as an alternative possibility in adult hepatocytes (Wakelam *et al.*, 1986).

The contribution of glucose to glycogen formation *de novo* was about 60 % after 2 days of culture, a value implying that part of glycogen was synthesized from glucose-1-phosphate-derived gluconeogenic substrates. Among the latter, glycerol, fructose, serine and glycine are possible candidates. However, lactate and amino acids, which enter at the level of pyruvate, are excluded, because the step mediated in adult liver by phosphoenolpyruvate carboxykinase is not operative in cultured foetal hepatocytes (Menuelle *et al.*, 1988; Bismut & Plas, 1989). In the present study, the contribution of glucose to glycogen formation was increased by insulin and decreased by glucagon, with a dominance of glucagon when hormones were combined, as observed for glycogenesis. The insulin effect is probably related to its preferential stimulation of [¹⁴C]glucose incorporation into glycogen compared with other ¹⁴C-labelled gluconeogenic substrates (Menuelle *et al.*, 1988). The glucagon effect can be explained by the low value (40 %) of the glucose contribution to glycogen synthesized *de novo* during the first day of culture (Plas *et al.*, 1979), which constitutes the core of glycogen and is not directly accessible to glucagon action.

A dose-dependent cessation of the responses to insulin and glucagon has been observed after 4 h of hormone presence, although at this time insulin- or glucagon-treated foetal hepatocytes are respectively responsive to a glucose load or another glycogenolytic agent, such as adrenaline (Plas & Nunez, 1975; Plas *et al.*, 1979; Moncany & Plas, 1980). In the present study, cells were also fully responsive to the antagonistic hormone. So, with the simultaneous presence of both hormones, each at 10 nM, a maximal glycogenic or glycogenolytic response could be triggered by the expression of an insulin or glucagon effect in cells desensitized to the opposite hormone. This is in marked contrast with the dominant effect of glucagon revealed by simultaneous addition of the two hormones, a situation where desensitization to insulin had developed, although its early glycogenic response had not taken place. The dissociation of the desensitizations to the two hormone indicates that they influence different steps in the regulation of glycogen metabolism. A transient glucagon effect has also been shown in adult liver,

where the action of glucagon on hepatic glucose output declined with time (El-Refai & Bergman, 1979; Cherrington *et al.*, 1982; Komjati *et al.*, 1985). The time- and dose-dependent glucagon desensitization has been correlated with a drastic alteration in the stimulation of adenylate cyclase (DeRubertis & Craven, 1976; Heyworth & Houslay, 1983; Noda *et al.*, 1984; Komjati *et al.*, 1985). The fact that the adenylate cyclase system of cultured foetal hepatocytes, desensitized to glucagon, remains sensitive to other glycogenolytic agonists suggests that the blockage is proximal to the receptor (Plas & Nunez, 1975; Moncany & Plas, 1980). Insulin desensitization in this system is probably caused by a defect in a post-receptor event, since no decrease in the cellular insulin receptor concentration occurred, the absence of receptor down-regulation being characteristic of the foetal stage (Menuelle & Plas, 1981; Vinicor & Kiedrowski, 1982; Caliendo & Patel, 1983; Alvarez & Blazquez, 1987). It is not clear whether glucagon receptor down-regulation occurs in foetal hepatocytes (Moncany & Plas, 1980). The decrease in cell-surface binding observed for insulin and glucagon after previous exposure to the native hormone corresponds to a new equilibrium resulting from receptor internalization, which is well-documented in the case of insulin (Soubigou *et al.*, 1986). No interference could be seen during the antagonistic effects of insulin and glucagon between the cell-surface binding of the two hormones.

Intermittent exposure (10 min on/10 min off intervals) to increasing glucagon doses elicited a glycogenolytic response identical with that obtained with continuous exposure, though the total amount of glucagon in the former was only 50 % of the latter. These findings are in line with the fact that glucagon concentrations lowered by pulsatile administration to perfused rat hepatocytes still give a similar glucose production to that with continuous administration (Weigle *et al.*, 1984). Also, insulin given continuously or intermittently increased glycogen labelling to the same extent in cultured foetal hepatocytes. Such a situation has been previously linked to the insulin receptor cycle induced by exposure to the hormone, whose revolution time averages 5–10 min (Soubigou *et al.*, 1986), a frequency found to be identical in perfused adult hepatocytes (Goodner *et al.*, 1988). Three exposures to insulin at 10 min on/10 min off intervals were sufficient to obtain a maximal stimulation of glycogenesis by insulin after 1 h, which corresponds to three rounds of insulin receptor cycles and is characterized by the synchrony between exposure to the hormone and the return of the receptors to the cell surface (Soubigou *et al.*, 1986).

The antagonistic effects of insulin and glucagon also developed in the same way using either the continuous or the intermittent mode when the hormones were added together. However, when cells were exposed to increasing doses of glucagon plus 10 nM-insulin alternating with exposure to one hormone alone, the resulting effect depended on which hormone was continuously present. Under these conditions, the continuous presence of glucagon or of insulin was more potent than intermittent exposure in antagonizing the effect of the opposite hormone. In isolated perfused rat liver, insulin given intermittently lowered the insulin concentration necessary to obtain equipotent inhibition of glucagon-stimulated hepatic glucose production when intervals between the 3 min exposures to insulin were less than 12 min. This reveals the importance of the duration of hormone exposure (Komjati *et al.*, 1986). The duration of the periodic pulses, which were of 10 min on/10 min off intervals, was chosen to relate to the kinetics of the insulin receptor cycle in cultured foetal hepatocytes.

In late gestation, the insulin/glucagon molar ratio is high in the rat foetus, with very high levels of insulin and glucagon (Girard *et al.*, 1974), as compared with the adult, where the mechanism of pancreatic secretion is pulsatile (Lefèvre *et al.*,

1987; Weigle, 1987). Despite the high concentrations of glucagon, glycogen is synthesized and stored in the late-fetal liver, which is considered to be 'resistant' to glucagon (Blazquez *et al.*, 1976; Vinicor *et al.*, 1976; Moncany & Plas, 1980). At birth, the insulin/glucagon molar ratio decreases dramatically and remains low during the first few hours of extra-uterine life, the fall in plasma insulin being considered as the prime factor involved in triggering glycogen mobilization (Girard *et al.*, 1973; Portha *et al.*, 1976; Snell & Walker, 1978). The present study shows that at least three factors are critical for the antagonistic effects of insulin and glucagon in cultured foetal hepatocytes on glycogen formation and mobilization: namely, the insulin/glucagon molar ratio, pre-exposure of cells to either insulin or glucagon (both time- and dose-dependent), and the mode of exposure (intermittent, continuous, or alternating). These three variables can result in completely different results regarding the regulation of glycogen metabolism, despite equivalent loads of the two hormones being administered.

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