Modulation of glucagon-induced glucose production by dexfenfluramine in rat hepatocytes

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The mechanism of the antihyperglycaemic action of dexfenfluramine (DEXF) was investigated in isolated rat hepatocytes exposed to glucagon. Preincubation of hepatocytes with DEXF caused a dose-dependent inhibition of cyclic AMP formation by 100 nM glucagon (K_i = 0.29 mM) that was almost complete at 1 mM DEXF. Surprisingly, glucagon-induced phosphorylase activation was not affected by DEXF despite the significant drop in cyclic AMP levels. Glucose production stimulated by glucagon was inhibited by up to 48% by 1 mM DEXF, and the rate of glucose production correlated positively with the steady-state concentration of glucose 6-phosphate. DEXF also partially restored lactate + pyruvate production which was abolished by an optimal concentration of glucagon. Although DEXF was not able to prevent the inactivation of pyruvate kinase by glucagon, the lack of further accumulation of phosphoenolpyruvate in DEXF-treated cells supports the conclusion that the flux through pyruvate kinase is stimulated, probably via the increase in fructose 2,6-bisphosphate, thereby increasing glycolysis. Our results thus indicate that DEXF counteracts the inhibition of glycolysis by glucagon and that this property might contribute to the antihyperglycaemic effect of this drug. Furthermore, this study shows that, in the presence of the drug, glucagon caused phosphorylase activation and pyruvate kinase inactivation without a significant increase in cyclic AMP levels.

INTRODUCTION

The *d*-isomer of fenfluramine, or dexfenfluramine (DEXF), is known to reduce high blood glucose levels, to improve glucose tolerance and to reduce insulin resistance in obese and diabetic patients [1–3]. These effects are independent of its anti-obesity action [1], which is believed to be centrally mediated through a serotoninergic mechanism (reviewed in [4]). Therefore distinct mechanisms may be involved in the central and peripheral effects of the drug. Previous studies have shown that DEXF affects lipid and glucose metabolism in adipose tissue as well as in liver [5–7]. Similarly to insulin, this antihyperglycaemic agent has been shown to act by increasing lipogenesis and inhibiting gluconeogenesis and ketogenesis at the hepatic level [8,9]. However, it is not known if DEXF exerts its antihyperglycaemic effects by inhibiting glycogenolysis and/or activating glycolysis. We therefore investigated the interaction of DEXF with glucagon-stimulated glucose production in isolated rat hepatocytes. We found that this drug inhibited several metabolic steps affected by glucagon, including cyclic AMP production, stimulation of glucose production and inhibition of glycolysis. These observations are consistent with the antihyperglycaemic effect of DEXF observed *in vivo*.

MATERIALS AND METHODS

Materials

Glucose, collagenase, defatted BSA, phosphoenolpyruvate (PEP), fructose-6-phosphate kinase (pyrophosphate-dependent) and analytical grade chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Glucagon was obtained from Lilly (Indianapolis, IN, U.S.A.). [^{3}H]Cyclic AMP was purchased from du Pont (New England Nuclear). Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR, U.S.A.). Auxiliary enzymes for metabolite measurements were obtained from Boehringer (Mannheim, Germany). DEXF was generously provided by Servier Canada.

Animals and cells

Male Wistar rats (200–250 g) were housed for 1 week in the Department's animal quarters prior to experimentation and were fed *ad libitum* with Purina Rat Chow (Charles River). Hepatocytes were prepared as previously described [10]. The viability of the hepatocytes was assessed by hormone responsiveness, by measuring the concentration of ATP and by Trypan Blue exclusion (85–95%). The cells (50 mg/ml) were preincubated for 30 min at 37 °C in a Krebs–Henseleit medium containing 1% defatted BSA with 10 mM glucose, with or without DEXF dissolved in double-distilled water, and were further incubated with the indicated concentrations of glucagon. After different incubation times, the reaction was stopped by the addition of 200 μl of ice-cold perchloric acid (12%, v/v), either to 1 ml of the cell suspension for the determination of cyclic AMP, ATP, ADP, glucose, lactate and pyruvate, or to the hepatocyte pellet after removing the medium by centrifugation (as recommended in [11]) for determination of glucose 6-phosphate levels. For phosphorylase *a* determinations, 100 μl of the cell suspension was frozen in liquid nitrogen and kept at −80 °C pending further determinations.

Enzyme and metabolite assays

Metabolite concentrations (glucose, lactate, pyruvate, ATP and ADP) were determined in duplicate in the neutralized deproteinized extract by standard enzymic procedures [12,13].

Abbreviations used: DEXF, dexfenfluramine; PEP, phosphoenolpyruvate; F-2,6-BP, fructose 2,6-bisphosphate.

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PEP and glucose 6-phosphate were assayed as described in [12], except that NADH and NADPH respectively were measured by fluorometry. Phosphorylase *a* was assayed in duplicate samples at 30 °C in homogenates of thawed cell suspensions as described by Hue et al. [14]. Pyruvate kinase was assayed in cell suspensions previously precipitated with ammonium sulphate in order to separate possible allosteric effectors [15]. Cyclic AMP was measured [16] in duplicate in neutralized perchloric acid extracts purified as described previously [17]. Fructose 2,6-bisphosphate (F-2,6-BP) was assayed as described in [18] in NaOH-treated cell suspensions which were subsequently heated at 80 °C for 5 min.

Calcium measurements

For calcium determinations in cell doublets or triplets, freshly isolated hepatocytes (3×10^5 per Petri dish) were plated on to collagen-coated coverslips in 1.5 ml of Williams E medium supplemented with 25 mM sodium bicarbonate, 5000 units/ml penicillin, 5 mg/ml streptomycin and 1 % BSA. The plates were incubated in a thermostatted O_2/CO_2 (95 %/5 %) incubator for 1 h. The incubation medium was then removed and 1 ml of the same Williams E buffer, pH 7.4, containing 10 mM Hepes without sodium bicarbonate was added to the plates. The cells were loaded with 2.5 μM fura-2 acetoxymethyl ester, supplemented with fetal bovine serum (5 %, v/v) and further incubated at room temperature with or without 1 mM DEXF. The intracellular calcium concentration was then measured using a DeltaScan 4800 dual monochromator spectrofluorimeter (Photon Technologies, London, Ontario, Canada) at an excitable wavelength of 340 nm and at 380 nm as a reference as previously described [19].

Expression of results and statistical analysis

Enzyme activities and metabolite concentrations are expressed per g wet wt. of liver. Data are representative of the indicated numbers of hepatocyte preparations. Averaged data are expressed as means ± S.E.M. Significant differences between groups of data were determined by using ANOVA (analysis of variance) and a paired Bonferroni test as appropriate.

RESULTS

Figure 1 shows that DEXF added to hepatocytes 30 min before addition of 100 nM glucagon caused a dose-dependent inhibition of cyclic AMP production ($K_i = 0.29$ mM) that was almost complete at 1 mM DEXF. Cyclic AMP levels determined in the presence of the optimal dose of DEXF (1mM) and glucagon were not significantly different from those measured under control conditions (2.12 ± 0.51 versus 0.55 ± 0.30 nmol/g of liver respectively). This suppression of cyclic AMP by 1 mM DEXF was maintained for up to 15 min (results not shown), but did not, however, prevent the activation of phosphorylase by glucagon (Figure 2). Phosphorylase activities and cyclic AMP levels were not different from control values after a 30 min preincubation of hepatocytes in the presence of DEXF without subsequent addition of glucagon.

Because of the discrepancy between the cyclic AMP and phosphorylase responses to glucagon and DEXF, we investigated whether the absence of a substantial increase in cyclic AMP levels affected other parameters such as glucose and lactate + pyruvate production. In order to be able to measure glucose production, the preincubation medium containing 10 mM glucose was replaced with fresh medium. It was verified that, under these conditions, the effects of DEXF on glucagon-induced

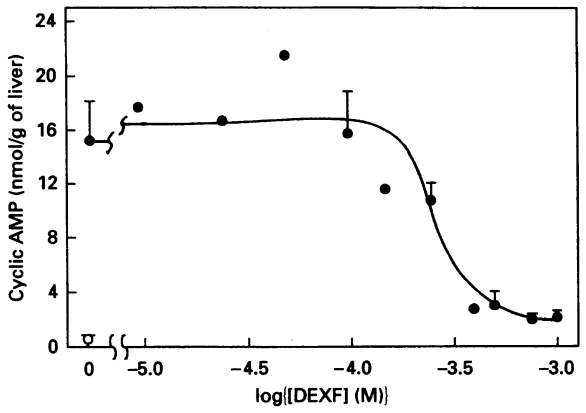

Figure 1 Effect of DEXF on cyclic AMP production in hepatocytes

Hepatocytes were preincubated in a Krebs–Henseleit medium containing 10 mM glucose for 30 min in the presence of the indicated concentrations of DEXF and were then challenged (●) or not (○) with 100 nM glucagon. Cyclic AMP production was assayed 2 min later. Results are expressed as mean values ($n = 2$) or means ± S.E.M. ($n = 3$–8).

phosphorylase activation and cyclic AMP production were maintained (results not shown). Figure 3(a) shows that 10 nM glucagon enhanced the rate of glucose production by more than 4-fold in hepatocytes, whereas preincubation with DEXF alone was without significant effect on this parameter. In cells preincubated for 30 min with 1 mM DEXF, the stimulatory effect of 10 nM glucagon on glucose production was progressively inhibited up to 48 % during the later stages of the incubation (10–15 min).

Lactate + pyruvate accumulation (Figure 3b) was suppressed by glucagon, presumably as a consequence of the inactivation of pyruvate kinase (Figure 4a), as indicated by the increase in PEP (Figure 4b). In DEXF-treated cells, where cyclic AMP

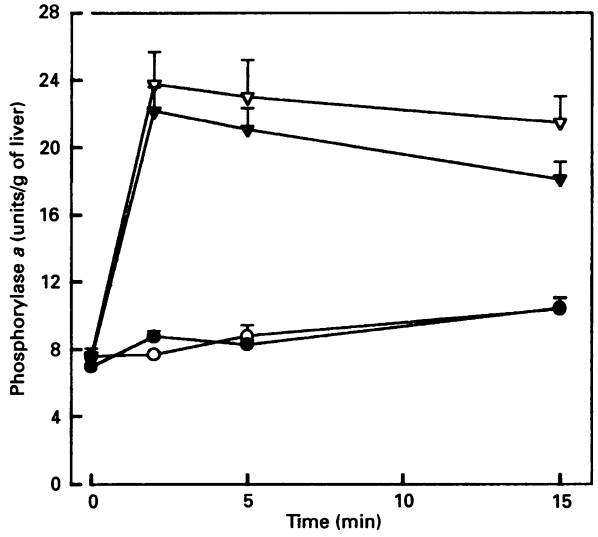

Figure 2 Effect of DEXF on the time course of phosphorylase activation in isolated hepatocytes

Hepatocytes were preincubated in a Krebs–Henseleit medium containing 10 mM glucose for 30 min with (●, ▼) or without (○, ▽) 1 mM DEXF. The cells were then challenged with 100 nM glucagon (▼, ▽). Results are expressed as mean values ± S.E.M. ($n = 3$).

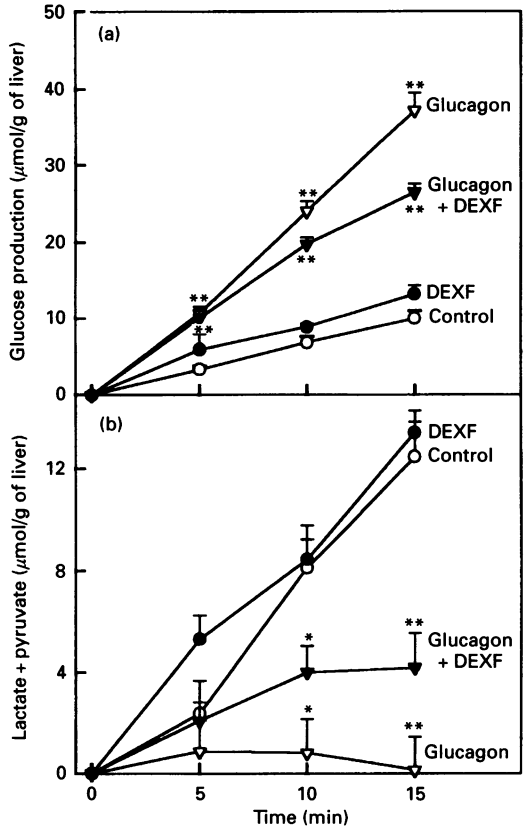

Figure 3 Effects of glucagon and DEXF on the time course of glucose production (a) and lactate + pyruvate accumulation (b) in isolated hepatocytes

Hepatocytes were preincubated as described in the legend to Figure 2. The cells were centrifuged and further incubated in the same medium without glucose and DEXF for the indicated times in the absence (○, ●) or presence (▽, ▼) of 10 nM glucagon. The incubation was stopped by the addition of perchloric acid; glucose, lactate and pyruvate concentrations were measured as described in the Materials and Methods section. Results are expressed as mean values ± S.E.M. ($n = 3$–4). Statistical significance is indicated for differences between glucagon + DEXF and glucagon alone, and for differences between control and glucagon alone (*$P < 0.05$; **$P < 0.01$).

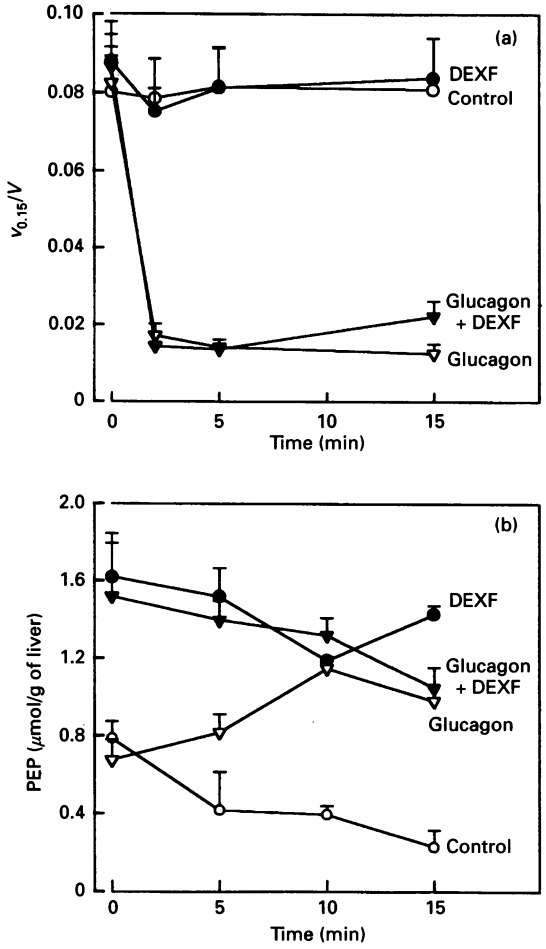

Figure 4 Effects of glucagon and DEXF on pyruvate kinase activity (a) and PEP levels (b)

Hepatocytes were preincubated as described in the legend to Figure 2 and were then challenged (▽, ▼) or not (○, ●) with 10 nM glucagon. Pyruvate kinase activity (**a**) was measured as described in the Materials and methods section. The active form of pyruvate kinase activity is measured by the ratio $v_{0.15}/V$, where $v_{0.15}$ and V are the activities measured at 0.15 and 5 mM PEP respectively. Results are expressed as mean values ± S.E.M. ($n = 3$). PEP (**b**) was assayed as described in the Materials and methods section after different incubation times in the indicated conditions. Results are expressed as mean values ± S.D. ($n = 2$).

levels are basal, lactate + pyruvate production that was suppressed by glucagon was partially restored (Figure 3b), but pyruvate kinase was still inactivated by glucagon (Figure 4a). DEXF added alone 30 min previously did not significantly affect subsequent lactate + pyruvate production (Figure 3b), but increased PEP levels (zero time in Figure 4b). In the presence of DEXF, glucagon did not increase PEP further (Figure 4b); rather, consistent with the restoration of glycolysis, PEP levels were seemingly decreased under these conditions. Furthermore, DEXF pretreatment of the cells resulted in higher F-2,6-BP levels (zero time, Figure 5), which remained significantly more elevated after the addition of glucagon than in control cells exposed to the hormone (Figure 5). This effect was noted within the first 2 min of incubation. After 15 min, the F-2,6-BP concentration was virtually zero in the presence of glucagon alone, whereas significant levels were still measured in the presence of both glucagon and DEXF.

Glucose 6-phosphate was increased more than 8-fold by glucagon alone (Table 1), but in DEXF-treated cells glucagon provoked only a 2-fold increase in glucose 6-phosphate levels. The presence of the drug alone during the preincubation did not change significantly the cell glucose 6-phosphate levels. The hepatocyte glucose 6-phosphate concentration was not consistent with the amount of phosphorylase *a* under the same experimental conditions, but was correlated positively and linearly with the rate of glucose production (Figure 6).

We also tested whether the observed effects of DEXF on glycogenolysis and glycolysis could be attributed to a change in ATP levels. We found that DEXF did not significantly affect the ATP content of the cells since, after 30 min of preincubation and a 15 min incubation with or without glucagon, the cellular ATP concentration remained stable (+DEXF, 3.36 ± 0.21 and 3.73 ± 0.11; −DEXF, 3.60 ± 0.28 and 3.25 ± 0.46 μmol/g of liver). Moreover, Figure 7 shows that there were no significant differences in the ATP/ADP ratios in the presence and absence of DEXF.

Finally, because glucagon was still able to exert its full effects on parameters such as phosphorylase and pyruvate kinase despite a lack of a significant increase in cyclic AMP, we verified whether

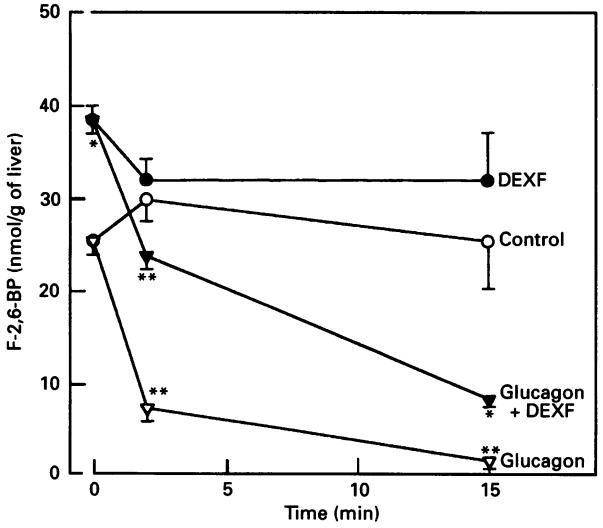

Figure 5 Effects of glucagon and DEXF on the time course of F-2,6-BP production in isolated hepatocytes

Hepatocytes were preincubated as described in the legend to Figure 2. The incubation following the addition of 10 nM glucagon (▽, ▼) was stopped at the indicated times by the addition of 100 μl of cell suspension to 300 μl of 25 mM NaOH. F-2,6-BP levels were determined as described in the Materials and methods section. Results are expressed as mean values ± S.E.M. ($n = 3$). Statistical significance is indicated for differences between glucagon + DEXF and glucagon alone, control and glucagon alone, and DEXF and control (*$P < 0.05$; **$P < 0.01$).

Table 1 Effects of glucagon and DEXF on glucose 6-phosphate levels

Hepatocytes were preincubated as described in the legend of Figure 2. Glucose 6-phosphate was measured as described in the Materials and methods section after a further 15 min incubation under the indicated conditions. Results are expressed as mean values ± S.E.M. ($n = 3$). Statistical significance is indicated for differences between control and glucagon alone and between glucagon + DEXF and glucagon alone (*$P < 0.01$).

	Glucose 6-phosphate (μmol/g wet wt. of liver)
Control	0.649 ± 0.090
DEXF (1 mM)	0.994 ± 0.297
Glucagon (100 nM)	4.168 ± 0.760**
Glucagon (100 nM) + DEXF (1 mM)	1.908 ± 0.550**

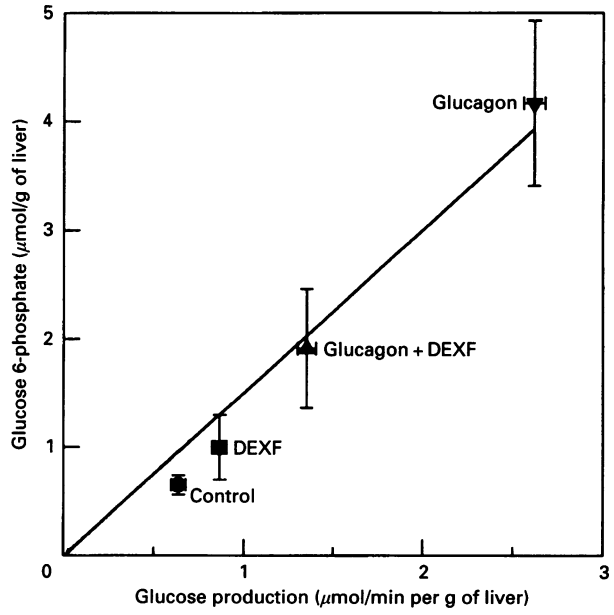

Figure 6 Correlation between glucose production and glucose 6-phosphate levels

Glucose production and glucose 6-phosphate measurements are described in legends of Figure 3 and Table 1 respectively. Rates of glucose production were calculated between 10 and 15 min of incubation.

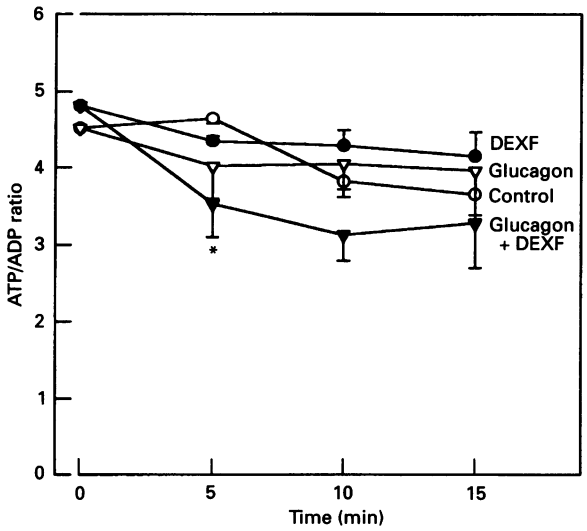

Figure 7 Effects of glucagon and DEXF on the ATP/ADP ratio in hepatocytes

Hepatocytes were preincubated as described in the legend of Figure 2. Cells were then incubated with (▽, ▼) or without (○, ●) 10 nM glucagon. ATP and ADP levels were measured as described in the Materials and methods section under the indicated conditions. Results are expressed as mean values ± S.E.M. ($n = 3$). Statistical significance is indicated for differences between control and glucagon + DEXF (*$P < 0.05$).

calcium could be an important component in the action of glucagon in the presence of DEXF. Figure 8 shows that glucagon is able to increase intracellular calcium in the absence or presence of DEXF (Figures 8a and 8b respectively). The calcium signal in cells preincubated with DEXF is increased from 190 nM to 240 nM, i.e. more than twice the increase obtained with glucagon alone (110 nM to 128 nM).

DISCUSSION

Role of cyclic AMP in the activation of phosphorylase and inactivation of pyruvate kinase by glucagon

The property of DEXF of inhibiting cyclic AMP production by optimal concentrations (10–100 nM) of glucagon has been used in this study to evaluate the role of this messenger in glucagon action. Several studies have reported that a decrease in cyclic AMP production does not significantly affect the metabolic effects of glucagon [20–22]. The present study reports that, at optimal concentrations, glucagon can activate phosphorylase, inactivate pyruvate kinase and enhance glucose production in hepatocytes without a significant increase in cyclic AMP. It is now well established that calcium plays a significant role in the

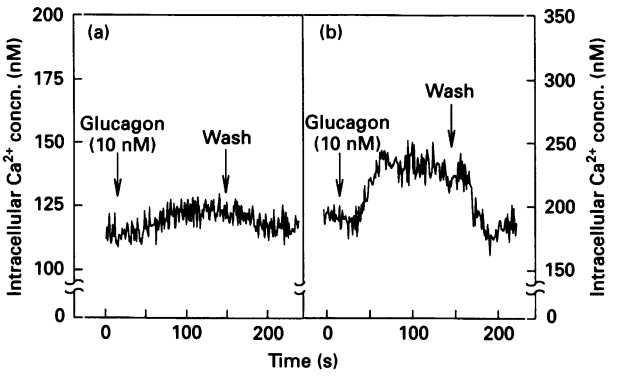

Figure 8 Effects of glucagon and DEXF on the intracellular calcium concentration

Plated hepatocytes were pretreated (**a**) or not (**b**) with DEXF under the conditions indicated in the Materials and methods section. A cell doublet or triplet was chosen from each coverslip and perfused with Krebs–Henseleit buffer supplemented or not with DEXF and/or 10 nM glucagon as indicated. The results shown are representative of hormone challenges performed in 3–6 hepatocyte doublets or triplets with 10 nM glucagon or 10 nM glucagon + 1 mM DEXF.

action of glucagon (reviewed in [23]). Therefore the activation of phosphorylase and inactivation of pyruvate kinase by glucagon in the presence of DEXF may be due in part to the calcium component of the signal, as the near-suppression of glucagon-induced cyclic AMP production does not result in inhibition of phosphorylase activation or of pyruvate kinase inactivation. Our results clearly support this notion, since the glucagon-induced increase in intracellular calcium not only still occurs in the presence of DEXF but is actually enhanced by the drug. In experiments using 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity, DEXF was still able to counteract induction of cyclic AMP production by glucagon, indicating that DEXF does not exert its effect by activating a phosphodiesterase (results not shown). Also, it is unlikely that this drug acts by perturbing hormone–receptor interactions non-specifically, since glucagon was still able to activate phosphorylase, inactivate pyruvate kinase in the presence of DEXF and increase intracellular calcium. Recently, the cloning of the glucagon receptor [24] has shown that different transduction signals (cyclic AMP and calcium) are mediated via the same receptor. Our results suggest that DEXF could inhibit adenylate cyclase or act via a partial inhibition of the stimulatory GTP-dependent protein G_s. Experiments performed with forskolin indeed indicate that DEXF does not affect cyclic AMP production induced by this agent (results not shown), thereby suggesting that DEXF probably affects G_s. Further studies on the action of DEXF will enable us to clarify the contributions of the different components (calcium and cyclic AMP) of the glucagon effect in the signal transduction cascade.

Mechanism of inhibition of hepatocyte glucose production by DEXF

It has been shown that DEXF is able to inhibit hepatic gluconeogenesis [8,9], but its antihyperglycaemic effects could involve other pathways such as glycogenolysis and glycolysis. Our results show that DEXF, like insulin, markedly inhibited glucagon-induced cyclic AMP production and significantly counteracted the stimulation of glucose production by glucagon. Since the drastic fall in cyclic AMP levels did not prevent phosphorylase activation even after 15 min of incubation, at which time cyclic AMP levels were maintained at a basal level, the decrease in glucose production is rationalized mostly by a stimulation of glycolysis rather than by a significant decrease in glycogenolysis. A stimulation of glycolysis is indicated by the partial restoration of lactate + pyruvate production by DEXF in the presence of glucagon and is probably a direct consequence of higher levels of F-2,6-BP, a potent stimulator of glycolysis.

The high PEP concentration in hepatocytes preincubated with DEXF remains unexplained. It might be due to a transient (basal values being the same at the end of preincubation with or without DEXF) inactivation of pyruvate kinase by DEXF during the preincubation. It is not, however, due to an inhibition of the active form of the enzyme, because if this were the case the subsequent lactate + pyruvate production would be inhibited by DEXF, which it is not. Elevated PEP levels in DEXF-treated cells were not further increased by the inactivation of pyruvate kinase by glucagon, but were actually decreased. This correlates with the enhanced lactate + pyruvate production, indicating that flux through the pyruvate kinase step was increased under these conditions. Different explanations are possible for this increased flux. One might be that DEXF allosterically stimulates pyruvate kinase by lowering the cytosolic ATP/ADP ratio, as it is known that pyruvate kinase is extremely sensitive to fluctuations in this ratio [25]. The second, and most probable, explanation (since we showed that DEXF does not alter the cellular ATP/ADP ratio) is the accumulation of fructose 1,6-bisphosphate, which may relieve the inhibition caused by glucagon inactivation of pyruvate kinase as previously reported [26]. High fructose 1,6-bisphosphate levels could result from the stimulation of phosphofructokinase by F-2,6-BP formation, a known consequence of low cyclic AMP levels [27]. Our results corroborate this hypothesis, as we show that the concentration of F-2,6-BP, which is progressively lowered by glucagon alone, is maintained at a significantly higher level in DEXF-treated cells where cyclic AMP is not significantly increased by glucagon.

As expected, glucagon alone increased glucose 6-phosphate levels. When the cyclic AMP signal was almost completely abolished by DEXF, glucose 6-phosphate formation by glucagon was decreased by 75%. The proposed stimulation of glycolysis in the presence of DEXF would explain the decrease in glucagon-induced glucose 6-phosphate levels despite a near-maximal phosphorylase activation. The tight correlation of glucose 6-phosphate levels with rates of glucose production suggests that DEXF inhibits glucose production by lowering glucose 6-phosphate levels.

The effect of DEXF in suppressing the glucagon-induced rise in cyclic AMP seems sufficient to drive the carbon flow from glycogen towards glycolysis, despite the fact that calcium may compensate for the lack of cyclic AMP for the action of glucagon on phosphorylase and pyruvate kinase. This effect of DEXF, combined with the previously documented inhibition of gluconeogenesis [8,9], probably contributes in large part to the antihyperglycaemic action of this drug.

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