EFFECTS OF VASOPRESSIN, GLUCAGON AND DIBUTYRYL CYCLIC AMP ON THE ACTIVITIES OF ENZYMES OF FATTY ACID ESTERIFICATION IN RAT HEPATOCYTES

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1. Introduction

Vasopressin and glucagon have a number of similar actions in rat liver. Both stimulate glycogenolysis and phosphorylase *a* activity (reviewed in [1]) and inhibit de novo lipogenesis [2–4]. In other respects, their actions are divergent. Thus vasopressin inhibits ketogenesis from oleate and stimulates oleate esterification, whereas glucagon opposes these effects [5]. The actions of glucagon are probably mediated via the cAMP system. Current opinion suggests that the hepatic effects of vasopressin are not mediated by changes in [cAMP] [1,6]. A requirement for Ca^{2+} in the action of the hormone has been indicated [1,7].

Stimulation of oleate esterification in hepatocytes could result from increases in the concentration of the substrates of esterification (fatty acyl-CoA, glycerol-3-phosphate, dihydroxyacetone phosphate). Vasopressin in the presence of oleate stimulated glycolytic flux and oleate removal in hepatocytes [5]. Another explanation is that the stimulation of oleate esterification is caused by an increase in the specific activity of the enzymes of esterification (GPAT and DHAPAT). The activity of GPAT (and possibly DHAPAT) is decreased in fat cells by starvation or adrenaline and is increased by insulin (reviewed in [8]). In the whole liver, GPAT activity is decreased by streptozotocin-diabetes, anti-insulin serum and Bu_2cAMP [9–11]. Furthermore, fat cell microsomal GPAT may be inhibited by phosphorylation by cAMP-dependent protein kinase [12]. Here, we describe the effects of vasopressin, glucagon and Bu_2cAMP upon the activities of enzymes of fatty acid esterification in isolated hepatocytes. We conclude that the alterations in enzyme activity which we observe can in part explain the rates of esterification seen in the presence of these agents.

Abbreviations: cAMP, adenosine 3',5'-cyclic monophosphate; Bu_2cAMP, dibutyryl cAMP; GPAT, glycerol-3-phosphate acyltransferase; DHAPAT, dihydroxyacetone phosphate acyltransferase; G-6-Pase, glucose-6-phosphatase; BSA, bovine serum albumin

2. Experimental

2.1. *Materials*

[Arginine] vasopressin (grade VIII) and glucagon were from Sigma, Poole, Dorset. All enzymes, coenzymes and substrates were from the same source or BCL, Lewes, East Sussex. Radiochemicals (including cAMP assay kits) were from the Radiochemical Centre, Amersham. Isolated hepatocytes were prepared from fed, anaesthetized (Nembutal, 60 mg/kg body wt) female rats as in [13,14]. Hepatocyte preparation commenced between 09:30 h and 10:30 h.

2.2. *Hepatocyte incubation and subcellular fractionation*

Hepatocyte incubation was done as in [15] in the presence of 1 mM oleate for 30 min in 40 ml total vol. Hormonal additions are described in the tables. Effectiveness of hormonal treatments was demonstrated by measurements of glucose and ketone body release. After incubation, hepatocyte suspensions were centrifuged (mark 3, MSE Minor bench centrifuge, 30 s,

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room temp.) and the pellets homogenized (20 up-and-down strokes, hand-held Teflon–glass Potter-Elvehjem homogenizer, radial clearance 0.1 mm) in 10 ml 10 mM Tris–HCl/192 mM mannitol/59 mM sucrose/1 mM EDTA/10 mM NaF (pH 7.4) (buffer A) at 0–4°C. The homogenate was centrifuged at 620 × g_{av} at 4°C for 10 min. The supernatant was decanted and centrifuged at 7250 × g_{av} for 10 min to produce a pellet (P1) and a supernatant (S1). The P1 pellet was washed once by resuspension in 10 ml buffer A and centrifugation at 9000 × g_{av} for 10 min. The pellet was resuspended in 1 ml buffer A and frozen in liquid N_2. The S1 fraction was centrifuged at 105 000 × g_{av} for 60 min, the pellet (P2) was resuspended in 1 ml buffer A containing 1 mM dithiothreitol and was frozen in liquid N_2.

Results of marker enzyme distributions in the hepatocyte subcellular fractions are shown in table 1. The P2 (microsomal) fraction was not markedly contaminated with citrate synthase (mitochondrial marker). The P1 (mitochondrial) fraction was extensively contaminated with arylesterase and G-6-Pase (microsomal markers) and may also contain peroxisomes and lysosomes [23]. The specific activities of the microsomal markers were about equal in both P1 and P2 fractions. Both P1 and P2 fractions were enriched in microsomes and the P1 fraction was enriched in mitochondria compared to the homogenate. The subcellular fractionation procedure is based on that used in [11]. It is not suggested that the procedure used here produces highly purified preparations of subcellular organelles. However, it was considered important to establish whether the relatively small changes in enzyme activities observed in homogenates were stable to subcellular fractionation and whether the changes were more apparent upon partial purification by fractionation.

2.3. *Enzyme and metabolite assays*

Enzymes were assayed at 30°C (except G-6-Pase which was assayed at 37°C) as follows by standard techniques: GPAT (EC 2.3.1.15) at 0.5 mM [U-^{14}C]-glycerol-3-phosphate (0.5 μCi/μmol) at either 1.75 or 6 mg/ml BSA [9], DHAPAT (EC 2.3.1.42) [11], citrate synthase (EC 4.1.3.7) [16], arylesterase (EC 3.1.1.2) [17]. G-6-Pase was assayed in 0.2 ml containing 50 mM imidazole/10 mM glucose-6-phosphate (pH 7.0) by measurement of P_i production [18]. For assay, extracts were standardized to 5 mg protein/ml by the biuret method [19]. Protein concentrations were subsequently assayed by the Lowry method [20]. Glycerol-3-phosphate was measured as in [21] and cAMP in KOH-neutralized $HClO_4$-treated hepatocyte extracts was measured by the Gilman [22] procedure using a kit from the Radiochemical Centre.

3. Results

3.1. *Activities of GPAT and DHAPAT in hepatocytes treated with vasopressin, glucagon and Bu_2cAMP*

Results are shown in table 2. For GPAT, assays were carried out at two BSA concentrations since BSA at 1.75 mg/ml has been reported to be optimal

Table 1
Activities of marker enzymes in hepatocyte subcellular fractions

Enzyme	Fraction	Enzyme activity (munit/mg protein)			
		Control	+10 nM vasopressin	Control	+10 μM Bu_2cAMP
Citrate synthase	Homogenate	81 ± 3 (8)	80 ± 5 (8)	89 ± 3 (6)	86 ± 4 (6)
	P1	114 ± 8 (11)	129 ± 5 (11)	110 ± 8 (6)	121 ± 7 (6)
	P2	13 ± 1 (11)	15 ± 1 (11)	18 ± 3 (6)	21 ± 2 (6)
G-6-Pase	Homogenate	87 ± 7 (7)	96 ± 13 (7)	92 ± 9 (5)	91 ± 7 (5)
	P1	196 ± 13 (7)	203 ± 16 (7)	184 ± 14 (6)	208 ± 22 (6)
	P2	195 ± 19 (7)	238 ± 25 (7)[a]	201 ± 24 (6)	172 ± 22 (6)[a]
Arylesterase	Homogenate	815 ± 43 (8)	828 ± 76 (8)	871 ± 58 (6)	842 ± 71 (6)
	P1	1567 ± 85 (10)	1650 ± 73 (10)	1577 ± 106 (6)	1727 ± 59 (6)
	P2	1685 ± 125 (10)	2168 ± 128 (10)[b]	1961 ± 231 (6)	1624 ± 161 (6)[a]

Enzyme activities were assayed as in section 2.3; 1 munit is 1 nmol substrate utilized/min; results are means ±SEM with no. obs. in parentheses; statistical significance, [a] $P < 0.01$; [b] $P < 0.001$; paired t-test

Table 2
Effects of vasopressin, glucagon and Bu_2cAMP on hepatocyte GPAT and DHAPAT activities

Fraction	[BSA] in assay (mg/ml)	GPAT activity (nmol glycerol-3-phosphate esterified . min^{-1} . mg $protein^{-1}$)					
		Control	+10 nM vasopressin	Control	+10 μM Bu_2cAMP	Control	+10 nM glucagon
Homogenate	1.75	1.51 ± 0.13 (13)	1.62 ± 0.17 (13)	1.71 ± 0.12 (11)	1.56 ± 0.11 (11)[a]	1.70 ± 0.24 (5)	1.52 ± 0.29 (5)
	6	1.77 ± 0.13 (13)	2.07 ± 0.20 (13)[b]	2.01 ± 0.14 (11)	1.72 ± 0.14 (11)[b]	2.00 ± 0.28 (5)	1.76 ± 0.32 (5)[a]
P1	1.75	2.04 ± 0.22 (6)	2.57 ± 0.27 (6)[c]	2.21 ± 0.18 (6)	2.46 ± 0.31 (6)	–	–
	6	2.89 ± 0.24 (10)	3.35 ± 0.34 (10)[b]	3.44 ± 0.26 (6)	3.64 ± 0.33 (6)	–	–
P2	1.75	2.16 ± 0.15 (10)	2.82 ± 0.18 (10)[c]	2.40 ± 0.30 (6)	1.98 ± 0.27 (6)[b]	–	–
	6	2.13 ± 0.36 (6)	2.68 ± 0.52 (6)[a]	2.33 ± 0.37 (5)	2.06 ± 0.26 (5)	–	–
		DHAPAT activity (nmol dihydroxyacetonephosphate esterified . min^{-1} . mg $protein^{-1}$)					
Homogenate	4	0.29 ± 0.03 (9)	0.30 ± 0.03 (9)	0.29 ± 0.03 (9)	0.27 ± 0.01 (9)	0.35 ± 0.09 (3)	0.32 ± 0.07 (3)
P1	4	0.55 ± 0.04 (6)	0.60 ± 0.05 (6)[a]	0.57 ± 0.04 (6)	0.54 ± 0.06 (6)	–	–
P2	4	0.25 ± 0.02 (6)	0.29 ± 0.03 (6)[a]	0.24 ± 0.02 (6)	0.22 ± 0.02 (6)	–	–

Hepatocytes were incubated and fractionated as in section 2.2; assays for GPAT were for 3 min and for DHAPAT 10 min; results are given as means ±SEM with the number of observations in parentheses; statistical significance, [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, paired t-test

for the microsomal isoenzyme whereas 6 mg/ml BSA is optimal for the mitochondrial isoenzyme [11]. The standard incubation time was 3 min and the assay was linear with protein concentration and time. Vasopressin significantly increased GPAT activities in homogenates compared to control values when assayed at 6 mg BSA/ml. When assayed at 1.75 mg BSA/ml, GPAT activities in the homogenate were not significantly increased by vasopressin. This BSA level is optimal for the microsomal isoenzyme [11] whose activity is increased by vasopressin (table 2). Presumably there may be factors in the homogenates which interfere with the GPAT assay. Changes in GPAT activities are generally larger on a % basis in subcellular fractions than observed in homogenates. Vasopressin also increased GPAT activities in the P1 and P2 fractions at both BSA concentrations. Increases in GPAT activity (assayed at 65 μM palmitoyl-CoA) were between 16–31%, the largest increase being observed in the P2 (microsomal) fraction at 1.75 mg BSA/ml. Although direct comparisons are invalid, a similar concentration of vasopressin increases esterification of oleate by 62% in rat hepatocytes [5]. DHAPAT activity is present in the fractions at only ~10–20% of GPAT values. In this case, differences are less clear. In the P1 and P2 fractions, there are small but significant DHAPAT increases observed in the presence of vasopressin compared to control values. Because DHAPAT may be mainly peroxisomal, it may therefore sediment in P1 [23].

Glucagon and Bu_2cAMP decreased GPAT activities in hepatocyte homogenates. Bu_2cAMP decreased GPAT activities in the P2 fraction (table 2). There were no significant effects of Bu_2cAMP or glucagon on DHAPAT activities (table 2). Bu_2cAMP decreased GPAT in the perfused rat liver and in microsomal fractions derived from that preparation [10]. Glucagon and Bu_2cAMP decreased oleate esterification in hepatocytes [24]. Glucagon also significantly ($P < 0.05$) decreased vasopressin-stimulated oleate esterification [5]. (No comment was made about this finding at the time.) We have also shown that 10 μM Bu_2cAMP decreased [1-^{14}C]oleate esterification in hepatocytes from 0.16 ± 0.02 to 0.10 ± 0.01 μmol . min^{-1} . mg wet wt hepatocytes^{-1} ($n = 7, P < 0.05$, Student's t-test).

In the course of the study of marker enzyme distribution, we observed that vasopressin significantly increased the specific activity of G-6-Pase in the P2 fraction by 22% and of arylesterase by 29%. Bu_2cAMP significantly decreased the specific activity of G-6-Pase in the P2 fraction by 14% and of arylesterase by 17%. These treatments did not significantly change the specific activities of the 3 marker enzymes in any other fraction. It should be emphasized that treatment with vasopressin or Bu_2cAMP did not alter marker enzyme activities in homogenates (contrast this with GPAT). We do not see any obvious reasons for these vasopressin or Bu_2cAMP induced changes in marker enzyme activities unless they selectively alter microsomal protein content.

When GPAT or DHAPAT activity in P2 is expressed relative to arylesterase or G-6-Pase activity, the significant differences between hormonal treatments disappear. The % changes in GPAT and DHAPAT activities are similar to those observed in marker enzyme activities in the P2 fractions. Other workers in this field have apparently encountered similar problems when attempting to relate GPAT activities to the activities of marker enzymes [9].

It is not possible to decide whether both mitochondrial and microsomal GPAT activities [25] are affected by vasopressin and Bu_2cAMP. Since P2 was relatively uncontaminated by mitochondria, it is considered that the microsomal GPAT activity was affected by vasopressin and Bu_2cAMP. Because P1 was contaminated by microsomes, vasopressin-induced alterations in GPAT could have been caused by microsomal contamination. However, when GPAT activity in P1 is expressed relative to G-6-Pase, there are significant ($P < 0.05, n = 6$) differences between control and vasopressin incubations (not shown). This may indicate effects of vasopressin on mitochondrial GPAT. Furthermore, GPAT activities in P1 were greater than in P2 when expressed relative to G-6-Pase or arylesterase activity. We consider that this shows the presence of mitochondrial GPAT in P1.

3.2. *Effects of vasopressin on hepatocyte cAMP and glycerol-3-phosphate concentrations*

Because adipose tissue GPAT is possibly regulated by a cAMP-dependent phosphorylation mechanism [11], effects of vasopressin on hepatocyte cAMP concentrations were investigated in the presence and absence of glucagon. There was no significant effect of vasopressin (10 nM) on basal cAMP concentrations in hepatocytes after 2–4 min exposure, nor was there any significant difference in cAMP concentrations between incubations (2–30 min) in the presence of a submaximal glucagon concentration (0.5 nM, cAMP raised 2–3-fold) and 0.5 nM glucagon + 10 nM vaso-

pressin. These results extend the observations of others who found that vasopressin did not have any effect on basal cAMP concentrations in the perfused liver [6].

Changes in fatty acid esterification could be mediated by changes in glycerol-3-phosphate concentration. There was no significant difference in glycerol-3-phosphate concentration between control and 10 nM vasopressin incubations (0.304 ± 0.031 vs 0.287 ± 0.039 μmol/g wet wt cells, respectively, mean ±SEM, $n = 4$). There was a significant decrease in glycerol-3-phosphate concentration between control and 10 nM glucagon incubations (0.304 ± 0.031 vs 0.174 ± 0.04, respectively, mean ±SEM, $n = 4$, $P < 0.05$ paired t-test). Similar effects of glucagon have been observed [26].

4. Discussion

Vasopressin increased the activities of GPAT and DHAPAT in rat hepatocyte homogenates or subcellular fractions. Glucagon and Bu_2cAMP decreased GPAT activities. We consider that these changes in enzyme activities may, at least in part, account for vasopressin-induced increases in fatty acid esterification in hepatocytes [5]. Glucagon or Bu_2cAMP-induced decreases in hepatic fatty acid esterification [5,10] may be caused by decreases in glycerol-3-phosphate concentrations and/or decreases in GPAT activity in hepatocytes. Although the changes in enzyme activities we observed were relatively small, they are comparable with those seen by other investigators in, e.g., streptozotocin-diabetes [9].

We presume that the effects of glucagon and Bu_2cAMP on fatty acid esterification could be mediated by cAMP-dependent protein kinase-mediated phosphorylation (and inhibition) of GPAT [12]. How vasopressin effects upon GPAT and DHAPAT activities are mediated is not known. Although Ca^{2+} have been implicated in the effects of vasopressin on glycogenolysis [1], they are not necessary for vasopressin-stimulated fatty acid esterification [7]. Under the latter conditions (minus external Ca^{2+}), vasopressin did not increase glucose release but did increase fatty acid esterification (not shown).

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