THE JOURNAL OF EXPERIMENTAL ZOOLOGY 279:62–70 (1997)

Glucagon and Glucagon-Like Peptide Signaling Pathways in the Liver of Two Fish Species, the American Eel and the Black Bullhead

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ABSTRACT Components of the signaling pathway for glucagon (GLU) and glucagon-like peptide-1 (GLP-1) were investigated in hepatocytes and membranes of two teleost fishes, the American eel (*Anguilla rostrata*) and the black bullhead (*Ictalurus melas*). Glucagon stimulated glucose release from isolated hepatocytes while increasing in a time- and dose-dependent fashion cAMP and inositol 1,4,5-trisphosphate (IP_3) concentrations. Phospholipase C (PLC), but not adenylyl cyclase (ACase), activities were statistically increased in hepatic membranes. GLP-1 also stimulated glucose release and a small but statistically significant increase in cAMP but not IP_3 concentrations in hepatocytes. The glucagon-family peptide also statistically stimulated PLC, but not ACase activities. Reponses to epinephrine (EPI) were generally similar with respect to glucose release and enzyme activation, but changes to cAMP were much greater than that of either GLU or GLP-1. These results support those in mammalian hepatocytes, which suggest that GLU may act through both the cAMP and the IP_3 signaling pathways. However, they do not provide further insight into the mechanism by which GLP-1 may exert its metabolic effects on the fish liver. Although small changes in cAMP were noted, the possibility of an IP_3 effect cannot be discounted. *J. Exp. Zool. 279:62–70, 1997.* © 1997 Wiley-Liss, Inc.

Glucagon (GLU), a hyperglycemic hormone in vertebrates, induces hyperglycemia in vivo (Morata et al., '82; Carneiro and Amaral, '83; de la Higuera and Cardenas, '86; Ottolenghi et al., '88b; Plisetskaya et al., '89; Mommsen et al., '92; Plisetskaya and Mommsen, '96) and stimulates glucose production in isolated hepatocytes (Birnbaum et al., '76; Klee et al., '90; Mommsen and Moon, '90; Brighenti et al., '91; Ottolenghi et al., '94; Plisetskaya and Mommsen, '96) in a number of fish species. Evidence for specific glucagon receptors on hepatic membranes has now been reported in two fish species, the American eel, *Anguilla rostrata*, and the brown bullhead (catfish), *Ictalurus nebulosus* (Navarro and Moon, '94), but evidence for these receptors is lacking in other species, including salmonids. However, the precise pathway between glucagon binding and the ultimate cellular effect of this hormone has not been extensively studied in fish

GLU increases hepatocyte cAMP content in a number of fish species (Mommsen et al., '87; Mommsen and Moon, '90; Puviani et al., '90; Brighenti et al., '91) and adenylyl cyclase (ACase) activities are stimulated in hepatic membranes (Ottolenghi et al., '88a). Glycogen phosphorylase activities are also reported to be stimulated by GLU, consistent with the increases in glucose production (Ottolenghi et al., '88b; Plisetskaya et al., '89; Puviani et al., '90; Brighenti et al., '91). In each case, stimulation required pharmacological levels of GLU and GLU was less potent than epinephrine (EPI), an observation contrary to that reported for mammalian liver (Ottolenghi et al., '88a). The question of activation of hepatic processes at low and presumably physiological GLU concentrations remains open.

Glucagon-like peptide (GLP), another member of the glucagon family of peptides and encoded on the glucagon gene (Plisetskaya, '90; Duguay and Mommsen, '94; Plisetskaya and Mommsen, '96), also stimulates glucose production in vivo (Plisetskaya

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Received 17 October 1996: revision accepted 27 March 1997

et al., '89) and in hepatocytes isolated from a number of fish species (Mommsen et al., '87; Mommsen and Moon, '89, '90; Puviani et al., '90; Brighenti et al., '91; Ottolenghi et al., '94). This hormone is more potent than GLU in many fish species (Plisetskaya and Mommsen, '96). Although GLP is present in mammals, it is generally believed not to affect hepatic metabolism (Blackmore et al., '91) with a single exception (Villanueva-Penacarrillo et al., '95), but does act at the level of the pancreas, adipose tissue, intestine, and brain (Wheeler et al., '93; Göke et al., '95). The binding of GLP to its specific receptor results in changes in intracellular cAMP and calcium concentrations in mammalian tissues (Wheeler et al., '93; Yada et al., '93), but only relatively small changes in hepatic cAMP in fish tissues when compared with those for GLU (Mommsen et al., '87; Mommsen and Moon, '89, '90). No specific hepatic GLP receptors have been identified in any fish species, although Navarro and Moon ('94) found that GLP poorly displaced ^{125}I-GLU from hepatocytes of eel and bullhead suggesting separate GLU and GLP receptors. The mechanism of action of GLP in fish hepatic metabolism remains elusive.

This study was initiated to more closely examine the signaling pathways for GLU and GLP in American eel and black bullhead (*I. melas*) hepatic tissues. These two species were used as hepatic tissue of both are known to significantly respond to GLU and GLP. Epinephrine is included in some experiments as previous studies (Fabbri et al., '95) have shown this hormone stimulates increased cAMP and inositol 1,4,5-trisphosphate (IP_3) concentrations in hepatocytes of these two species.

MATERIALS AND METHODS

Chemicals

[8-^{3}H]Adenosine 3',5'-cyclic monophosphate, ammonium salt (^{3}H-cAMP; sp act 20–30 Ci·mmol^{-1}) and D-myo-[^{3}H]-inositol-1,4,5-trisphosphate, potassium salt (^{3}H-IP_3; sp act 54 Ci·mmol^{-1}) were purchased from Amersham Canada (Oakville, ON), and L-3-phosphatidyl [2-^{3}H]-inositol 4,5-bisphosphate (^{3}H-PIP_2; sp act 8 Ci·mmol^{-1}) was purchased from NEN Research (Du Pont; Mississauga, ON). Hormones were obtained from various suppliers, but were all of the highest quality: EPI (Sigma), GLU (Lilly Research Laboratories, Indianapolis, Indiana; or Sigma Chemical, Milano, IT), and GLP (human GLP-1_{7-37}, Peninsula Laboratories, Belmont, CA). All other biochemicals were purchased either from Sigma Chemical (St. Louis, MO, or Milano, IT) or from local distributors; they were of the highest purity possible.

Experimental animals

American eels (*Anguilla rostrata*; 250–350 g) were captured in trapnets on the St. Lawrence River near Lancaster, Ontario, by a local supplier. These animals were transported to Ottawa on ice and held at 12°C. There were two suppliers black bullheads (*Ictalurus melas*) depending on the location of the experiment. For those experiments undertaken in Ottawa, bullheads (150–200 g) were supplied from cultured stock by Dr. Joe Buttner, State University of New York, Brockport, and transported in aerated holding tanks to Ottawa. For those experiments undertaken in Ferrara, bullheads (200–300 g) were obtained from a local dealer. In both cases, bullheads were held in aerated dechlorinated tapwater at 18–20°C. Eels were not fed during holding, but all eels were used within three months of capture. Bullheads were fed every second day to satiety. All experiments were carried out from June to December, unless indicated, and the fish were held on a 12:12 light/dark photoperiod.

Hepatocyte isolation and incubations

Hepatocytes were isolated from eel and bullhead by the collagenase perfusion method previously documented (Fabbri et al., '95). Cells were resuspended in a medium consisting of Hanks' supplemented with 1.5 mM $CaCl_2$ and 2% bovine serum albumin (BSA; Sigma) adjusted to pH 7.68 and to a cell weight of approximately 50 mg·ml^{-1}.

Hepatocytes were incubated in the presence or absence of hormone in a final volume of 200 µl (150 µl cell suspension, remainder hormone or Hanks', depending upon the experiment) at 18°C; specific conditions are listed in table and figure legends. The reaction was stopped by adding 25 µl perchloric acid (6% final concentration), and tubes were vortexed and kept on ice for 10 min before centrifugation (12,000 g for 5 min). Supernatants were neutralized with 1 M $KHCO_3$ and aliquots used for various assays.

Determination of second messengers, metabolites, and enzymes

Neutralized supernatants were assayed for cAMP and myo-inositol-1,4,5-trisphosphate (IP_3) by competitive protein-binding assays as previously reported (Fabbri et al., '95). Glucose was assayed colorimetrically and glycogen was assayed

as glucose following amyloglucosidase hydrolysis according to Mommsen and Moon ('90).

Adenylyl cyclase (ACase, EC 4.6.1.1) was assayed on a crude membrane fraction obtained from homogenizing hepatocytes in three volumes of water and after centrifugation at 18,000 g for 10 min at 4°C. The resulting pellet was resuspended in 50 mM tris-HCI, pH 7.4 to a final protein concentration of 1.5 $mg \cdot ml^{-1}$, as determined by the Lowry method (Lowry et al., '51). Membrane protein (150 µg) was incubated and the resulting cAMP formed after a 10-min incubation period at 30°C was assayed as previously reported (Ottolenghi et al., '88a). The concentration of GTP in the assay medium was 50 µM.

Phospholipase C (PLC) activities were measured with slight modifications of the method of Mazzoni et al. ('92). Briefly, after a 5 min pretreatment with hormone agonists, hepatocytes were centrifuged and the resulting pellet homogenized in 10 mM Tris-HCl, pH 7.0, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF); all procedures were carried out on ice. Samples (10 µl containing 50 µg protein) were incubated for 30 min at 22°C in a final volume of 100 µl containing ^{3}H-PIP_2 (30,000 dpm), 3 nM PIP_2, 0.06% Na-taurodeoxycholate, 0.6% NaCl, 0.1 mM $CaCl_2$, and 100 mM Tris-HCl, pH 7.0. The reaction was terminated by adding 2 ml of ice-cold chloroform: methanol: 37% HCl (200:100:0.75 v/v). After the addition of a further 0.5 ml of 0.6 N HC1, samples were vortexed and then centrifuged at 3,000 g for 10 min. Aliquots of the upper phase were transferred to scintillation vials and the water-soluble radioactivity was evaluated using a Beckman LS 1801 spectrometer.

Protein kinase A (PKA) was assayed according to Mehrani and Storey ('93) by following the incorporation of [γ-^{32}P]ATP into the synthetic peptide substrate kemptide at 18°C. Incubation time was 5 min, which was the time necessary to achieve maximum activation of the enzyme by hormones (data not shown). Enzyme activity is expressed as percent of activation, calculated as the ratio between the radioactivity measured in the absence and in the presence (100%) of cAMP.

Statistical analysis

All statistical tests were undertaken with SigmaStat (Version 2.0; Jandel Scientific Software). The particular ANOVA and post-hoc multiple comparison test or t-test selected by the program was accepted as the test of choice.

RESULTS

Glucose release from isolated hepatocytes

Glucagon (GLU), GLP, and EPI stimulated glucose release from hepatocytes isolated from bullhead (Tables 1, 2; Fig. 1). Release was dose-dependent (Fig. 1), with apparent activation by EPI occurring at lower hormone concentrations than either GLU or GLP; maximum responsiveness was similar for the three agonists. Glucose release was also stimulated by 10 µM forskolin (FSK), a compound which directly stimulates ACase. Release was not stimulated above values with GLU and GLP alone by adding either 10 mM theophylline (THEO) or 10 mM LiCl (Table 2), agents which reduce the breakdown of cAMP and IP_3, respectively. Unexpectedly, THEO actually decreased rather than increased GLP-stimulated glucose release at 15 min (Table 2).

Changes in cAMP and IP_3

Glucagon at various concentrations increased cAMP and IP_3 levels in a time-dependent fashion in hepatocytes isolated from both the eel and the bullhead (Tables 1, 2; Figs. 2, 3). Peak concentrations of IP_3 were reached within 60 sec of hormone addition and decreased toward control

TABLE 1. Changes in glucose production and cAMP content of hepatocytes isolated from the black bullhead (Ictalurus mela) *and incubated with various agonixts*

Condition	Glucose production $\mu mol \cdot g^{-1}$ cell	cAMP content $nmoles \cdot g^{-1}$ cell
Control	5.25 ± 0.65 (7)	1.10 ± 0.20 (7)
EPI–10 µM	30.1 ± 4.4 (7)*	9.13 ± 1.60 (7)*
GLU–1 µM	24.8 ± 3.3 (7)*	2.07 ± 0.45 (7)*
GLP–1 µM	25.0 ± 2.3 (6)*	1.69 ± 0.29 (7)*
FSK–10 µM	25.0 ± 3.3 (7)*	14.0 ± 3.6 (7)*

Experiments undertaken in Ferrara, Italy, and samples prepared as in Methods and Materials. Incubations were for 15 min at 18°C. Abbreviations: EPI, epinephrine; GLU, glucagon; GLP, glucagon-like peptide (7–37); FSK, forskolin. Values represent means ± SE.
*Significantly different from control at $P < 0.05$ (Kruskal-Wallis one-way ANOVA on ranks; post-hoc Dunnett's Method).

TABLE 2. Changes in glucose production and cAMP and IP3 contents of hepatocytes isolated from black bullheads (Ictalurus melas) *and incubated with glucagon (GLU; 1 μM) and glucagon-like peptide (GLP; 3 μM)*

Condition	Time: minutes	Glucose production μmol.g^{-1} cell	cAMP content nmol.g^{-1} cell	IP3 content nmol.g^{-1} cell
GLU	1	1.68 ± 0.39 (9)	1.26 ± 0.14 (9)	2.29 ± 0.91 (4)
	15	5.59 ± 1.20 (9)*	0.94 ± 0.13 (9)*	2.96 ± 1.7 (6)
GLU + THEO	1	2.48 ± 0.52 (8)	1.26 ± 0.27 (9)	not done
	15	4.31 ± 0.74 (9)*	6.87 ± 0.69 9()*,**	
GLU + LiCl	1	2.20 ± 0.47 (9)	not done	1.58 ± 0.49 (7)
	15	5.39 ± 1.07 (9)*		3.51 ± 1.4 (4)
GLP	1	1.08 ± 0.32 (7)	1.40 ± 0.17 (9)	1.16 ± 0.47 (6)
	15	3.94 ± 0.76 (9)*	0.70 ± 0.08 (9)*	1.09 ± 0.51 (6)
GLP + THEO	1	1.12 ± 0.26 (8)	1.35 ± 0.26 (9)	not done
	15	1.95 ± 0.55 (9)**	5.87 ± 0.57 (9)*,**	
GLP + LiCl	1	1.76 ± 0.74 (8)	not done	2.47 ± 0.62 (7)
	15	4.10 ± 1.05 (8)		1.56 ± 0.65 (7)

Incubation time was either 1 or 15 min and values are presented after subtraction of the appropriate control values. Experiments were undertaken in Ottawa during the month of February. Analysis as in Materials and Methods; concentrations of LiCl and theophylline (THEO) were 10 mM. Control values (either no addition or LiCl or THEO without hormone) within a time period were not statistically different from one another (Kruskal-Wallis One-Way ANOVA on ranks): values are 0.77 ± 0.10 (17) (1 min) and 4.77 ± 0.33 (24) (15 min) μmoles·g^{-1} cells for glucose production; 0.094 ± 0.02 (10) (1 min) and 0.24 ± 0.05 (14) (15 min) nmoles·g^{-1} cells for cAMP content; and, 0.55 ± 0.28 (5) (1 min) and 2.66 ± 0.91 (7) (15 min) nmoles·g^{-1} cells for IP_3 content

*Statistically different from 1 min value ($P < 0.05$, Mann-Whitney Rank Sums test or paired t-test, where appropriate).

**Statistically different from 15 min hormone treatment ($P < 0.05$, Kruskal-Wallis one-way ANOVA; post-hoc Dunnett's Method).

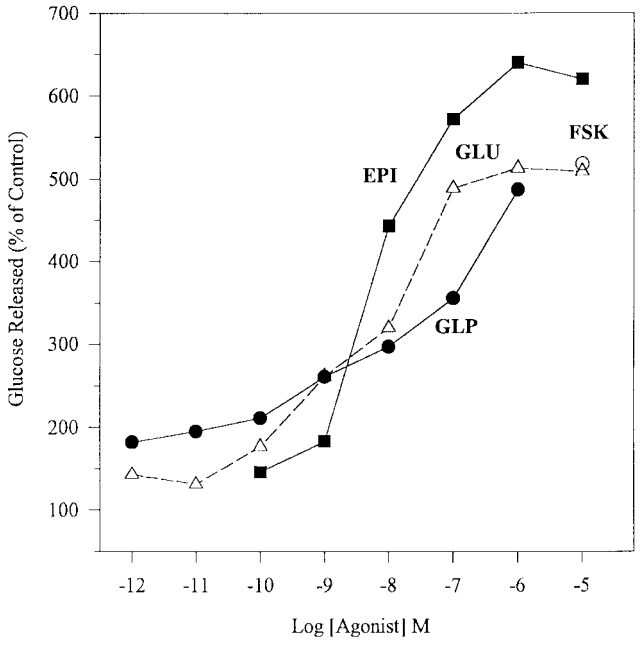

Fig. 1. Increase in glucose released from black bullhead (*Ictalurus melas*) hepatocytes incubated with varying concentrations of epinephrine (EPI, closed squares), glucagon (GLU, open triangles), glucagon-like peptide-1 (GLP, closed circles), or forskolin (FSK, open circle). Cells (approximately 50 mg·ml^{-1}) were incubated at 18°C for 15 min and incubations terminated as noted in Materials and Methods. Values represent the average change from control (no addition) of n = 7 experiments.

thereafter, while cAMP concentrations plateaued only after about 5 min (Figs. 2, 3). There was a major quantitative difference between the concentrations of cAMP achieved in the two species, with eel hepatocytes producing much higher concentrations than the bullhead hepatocytes. This was not the case for IP_3 levels; basal IP_3 levels in eel hepatocytes were statistically less than those for the bullhead, but the relative increase in IP_3 concentrations was similar in both species.

Hepatocyte cAMP and IP_3 contents were analyzed in two additional experiments. First, cAMP levels noted in Tables 1 and 2 are consistent with those reported in Figure 2A for bullhead. Interestingly, cAMP content decreased at 15 min in the presence of GLU (Table 2), but increased in the presence of theophylline in the same time period. Unlike values reported in Figure 3A, the second experiment showed no GLU-stimulated increase in IP_3 levels, even in the presence of LiCl (Table 2). The factor or factors responsible for this discrepancy in the ability of GLU to enhance IP_3 content are unknown. The GLU-stimulated increase in cAMP was dose-dependent (Fig. 4), and as noted in Figure 2, eels produced 4 times more cAMP than bullhead cells at 1 μM GLU. No statistically significant correlation was observed between dose and IP_3 production (data not shown).

A small, but statistically significant, increase in cAMP content occurred at μM concentrations of GLP in bullhead hepatocytes (Table 1); again,

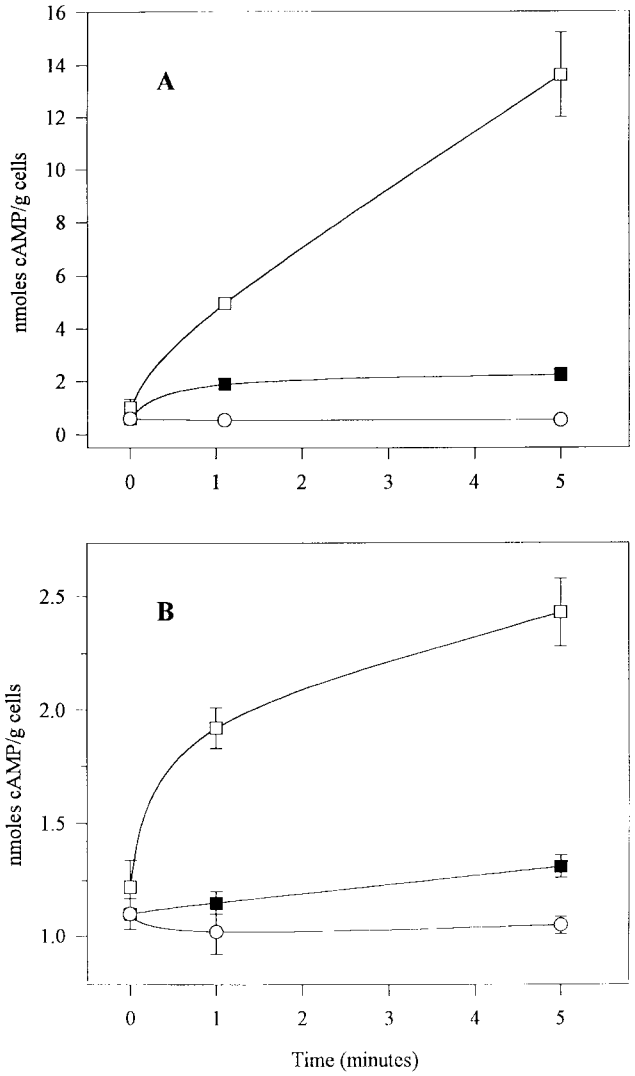

Fig. 2. Time course of cAMP production in hepatocytes isolated from American eel (**A**) and black bullhead (**B**) and incubated in the presence of glucagon (GLU, open squares, 100 nM; closed squares, 0.01 nM; open circle, control). All values are mean ± SEM, n = 3 independent experiments (where error bars not noted, they lie within the symbol). All increases are statistically different from control ($P < 0.05$, Kruskal-Wallis One-Way ANOVA; post-hoc Dunnett's Method) except the GLU-induced response at 1 min and 0.01 nM in bullhead.

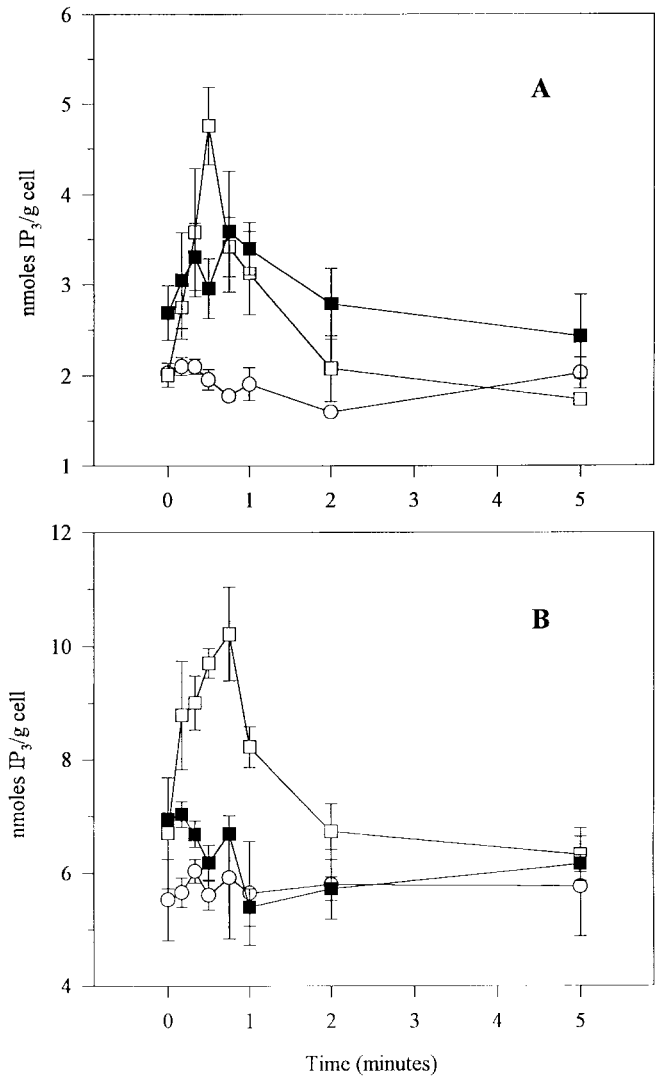

Fig. 3. Time course of IP_3 production in hepatocytes of American eel (**A**) and black bullhead (**B**) incubated in the presence of glucagon. Symbols and values as in Figure 2. Peak IP_3 concentrations were significantly different ($P < 0.05$, Krusal-Wallis One-Way ANOVA; post-hoc Dunnett's Method) compared to control, except for 0.01 nM GLU in bullhead.

as with GLU, cAMP content decreased at 15 min with GLP but increased by theophylline at 15 min (Table 2). Glucagon-like peptide did not elevate IP_3 content even with LiCl (Table 2).

Epinephrine (10 μM), presented as a control, increased cAMP content of bullhead hepatocytes (Table 1). The extent of this increase was 4–5 time greater than that for either GLU or GLP. This response was dose-dependent, with both GLU and GLP showing a lower activation constant than EPI, but even these were well above physiological hormone concentrations (data not shown). Stimulation by FSK was greater than by EPI.

Changes in enzyme activities

Adenylyl cyclase (ACase) activities were not statistically increased by GLU and GLP, although FSK stimulated activities in a highly significant manner (Fig. 5). Epinephrine at 10 μM statistically increased activities after 15 min compared to T_o but not T_{15}, due no doubt to the large variation in the values obtained. In addition, these hor-

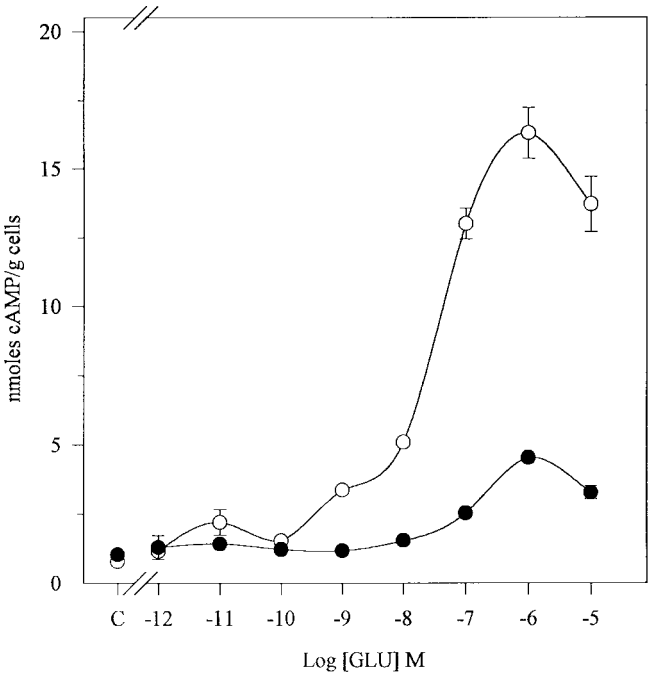

Fig. 4. Increase in cAMP production in hepatocytes of American eel (open circles) and black bullhead (closed circles) at various glucagon concentrations. Hepatocytes were incubated for 5 min at 18°C and cAMP assayed according to the Materials and Methods. Values are means ± SEM, n = 3 independent experiments. Activation constants (K_a = 18 ± 6 nM and 91 ± 59 nM for eel and bullhead, respectively) were calculated by Hill plots and were not significantly different.

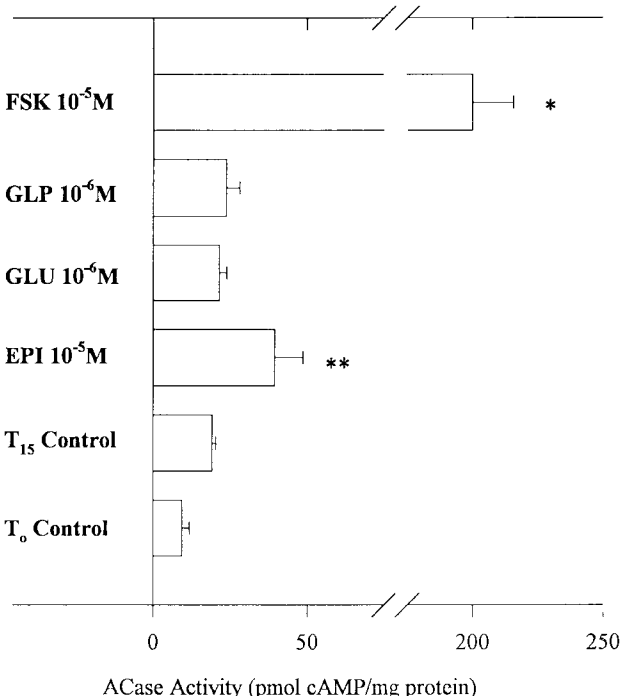

Fig. 5. Adenylyl cyclase (ACase) activities in crude membranes isolated from black bullhead hepatocytes and incubated for 15 min at 18°C in the presence or absence (control) of agonists. Levels of cAMP were estimated as described in Materials and Methods. Values represent means ± SEM, n = 6 (minimal); *statistically different from all other values; **statistically different from T_0 control ($P < 0.05$, Kruskal-Wallis One-Way ANOVA; post-hoc Dunnett's Method). Abbreviations: FSK, forskolin; GLP, glucagon-like peptide-1; GLU glucagon; EPI, epinephrine.

mones also stimulated PLC (Fig. 6), with optimal activities occurring between 2 and 5 min of incubation with agonist (data not shown). Each agonist activated to about the same level and showed similar activation values (K_a, 10^{-8} to 10^{-9} M agonist), a value below that noted for glucose release and cAMP production.

A preliminary experiment estimated cAMP-dependent PKA activities in two bullhead hepatocyte preparations. The active form of PKA was 40, 79, and 92% at 0, 1, and 10 µM EPI and 43, 69, and 65% at 0, 1, and 10 µM GLU.

DISCUSSION

Plisetskaya and Mommsen ('96) have recently reviewed our understanding of the actions of the glucagon-family peptides in fish species. It is clear that our understanding is at a very rudimentary level and that knowledge of the signaling processes of both GLU and GLP is controversial in mammals and fish. The present study was undertaken to overcome some of this misunderstanding. We demonstrate that GLU and GLP stimulate hepatocyte glucose production, and that this effect is associated with a GLU-induced increase in both cAMP and IP_3 in hepatocytes and a GLU and GLP-induced increase in PLC activities in hepatic membranes. Although not conclusive, this study provides evidence for both hormones acting on a multi-functional membrane receptor in fish hepatic membranes.

Although controversial, mammalian GLU and GLP receptors are thought to belong to a separate branch of the superfamily of G-protein-coupled receptors which are able to induce both cAMP and intracellular Ca^{2+} increases (Wheeler et al., '93; Hjorth et al., '94; Moens et al., '96). Whether the increase in $[Ca^{2+}]_i$ is phosphoinositol- (Wheeler et al., '93; Gromada et al., '95b) or voltage- (Gromada et al., '95a; Holz et al., '95) sensitive is far from clear. However, in most cases, these experiments have been undertaken using pancreatic cells, as GLP-1 is not thought to have a metabolic role in mammals (Plisetskaya and Mommsen, '96). Even with GLU, which has a metabolic effect,

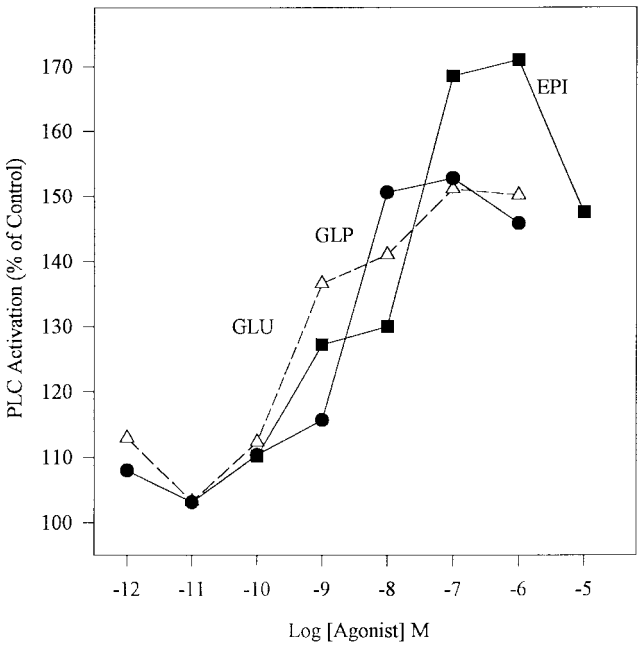

Fig. 6. Activation of phospholipase C (PLC) in hepatocytes isolated from black bullhead and incubated for 5 min with varying concentrations of epinephrine (EPI, closed squares), glucagon (GLU, open triangles), and glucagon-like peptide-1 (GLP, closed circles); assay as described in Materials and Methods. Control activities (no hormone) are (pmoles PIP_2 hydrolyzed·mg^{-1} protein): 1.31 ± 0.11, n = 5, EPI; 0.98 ± 0.05, n = 5, GLU; 0.92 ± 0.08, n = 4, GLP.

the role of Ca^{2+} is debated (Kraus-Friedmann and Feng, '96).

This study, however, clearly shows that glucagon stimulates glucose production and increases both cAMP and IP_3 content of bullhead hepatocytes. These effects are both time (Figs. 1, 2, 3) and concentration (Fig. 4) dependent, with statistically significant increases occurring even at 0.01 nM (approximately 0.03 ng/ml) GLU. The K_a(GLU) for cAMP increase (approximately 50 nM; Fig. 4) does exceed the reported concentrations of plasma GLU in these fish (2.3 and 0.13 ng/ml, bullhead and eel, respectively; Navarro and Moon, '94). A dose-response was not detected for the IP_3 response, but again, even at 0.01 nM GLU, IP_3 levels were significantly elevated above control (Fig. 3). Both theophylline and LiCl statistically increased GLU-stimulated glucose production, but this effect was reflected only in statistically elevated cAMP, not IP_3 concentrations (Table 2). The data reported in Table 2 are also interesting in that cAMP levels actually fell with GLU alone, implying an increased turnover of cAMP which had reached a maximum by 5 min (Fig. 2B). Further studies are required to examine second messenger turnover in these species. The inconsistencies, particularly in the GLU-IP_3 response, have been observed in mammalian hepatocytes, with some investigators reporting no changes in IP_3 (see Kraus-Friedmann and Feng, '96).

The major differences between the eel and bullhead cAMP and IP_3 responses to GLU (Figs. 2, 3) have also been reported in response to EPI (Fabbri et al., '95). The exact significance of these differences is unknown, although the two species were held under distinct nutrient conditions (bullheads, fed; eels, fasted for 4 months). Our studies also demonstrate that EPI and GLU stimulate glucose release to a similar extent (Fig. 1, Table 1), supporting previous studies on bullheads (Ottolenghi et al., '91, '94). However, at sub-nM concentrations, GLU was more effective than EPI (Fig. 1). Brighenti et al. ('91) did show that EPI was more effective at stimulating glucose release than GLU in both catfish and rainbow trout hepatocytes.

Glucagon did not significantly stimulate ACase activities in isolated membrane fractions (Fig. 5), as previously reported by Ottolenghi et al. ('88a). EPI was a more effective stimulator (Fig. 5), in line with the major difference observed in cAMP accumulated with GLU and EPI (Table 1). Forskolin, which stimulates ACase activities directly, caused a massive increase in the enzyme activities and a further increase in cAMP level. It is interesting, however, that these differences in ACase activities and cAMP levels are not reflected in significant differences in the amount of glucose actually released in the presence of GLU, EPI, and FSK (Fig. 1, Table 1).

Glucagon also significantly stimulated the activities of PLC (Fig.6), the enzyme which is linked to changes in cellular IP_3 concentrations (Kraus-Friedmann and Feng, '96). To the authors knowledge, these are the first such measurements of PLC in fish hepatocytes. We have previously shown that EPI increases IP_3 (Fabbri et al., '95) and $[Ca^{2+}]_i$ (Zhang et al., '92; Moon et al., '93) in bullhead hepatocytes. We have preliminary evidence (T.W. Moon, unpublished) that GLU induces a glucose-dependent change in $[Ca^{2+}]_i$, but further studies are needed to validate this link between GLU-PLC-IP_3. In fact, in mammalian hepatocytes there is some evidence that IP_3 concentrations do not change in the presence of GLU, and that the $[Ca^{2+}]_i$ changes noted are a result of cAMP-dependent PKA phosphorylation of the Ca^{2+} mobilization system (see Kraus-Friedmann and Feng, '96). Jelinek et al. ('93), using BHK (baby hamster kid-

ney) and COS-7 cells transfected with pLJ4 (a plasmid containing the rat glucagon receptor), did demonstrate changes in both IP_3 and $[Ca^{2+}]_i$ to GLU. This is an area of active research and one where the fish hepatocyte system could contribute, given that changes in PLC and IP_3 have been clearly demonstrated. In addition, given the short time periods over which IP_3 content increased, the experiments reported here may have missed the critical period over which IP_3 changes were significant.

Unfortunately, this study provides little new information on GLP actions in fish hepatocytes. GLP does increase glucose release as effectively as GLU (Fig. 1, Table 1), in contrast to other reports where GLP is generally more effective than GLU (Brighenti et al., '91; Plisetskaya and Mommsen, '96). GLP did not statistically increase ACase activities (Fig. 5), although a small but statistically significant increase in cAMP concentrations was noted (Table 1), which was further enhanced in the presence of theophylline (Table 2). There was no elevation in IP_3 concentrations (Table 2), even though PLC was activated (Fig. 6). These data support previous unsuccessful attempts to define the signal transduction system for GLP in the fish hepatocyte (see Plisetskaya and Mommsen, '96). Although GLP is not thought to be a metabolic hormone in mammals, there remains a controversy as to its precise signaling pathway. In the pancreatic β cell and certain cell lines, GLP-1 activates ACase activities and increases cAMP concentration (Gromeda et al., '95a,b; Holz et al., '95; Moens et al., '96). In addition, Wheeler et al. ('93) and Yada et al. ('93) have shown increases in IP_3 and $[Ca^{2+}]_i$ in rat pancreatic cells which contain GLP-1 receptors. However, others believe that this increase in $[Ca^{2+}]_i$ is indirectly related to changes in Ca^{2+} currents across the membrane as a result of cAMP-dependent changes (Gromeda et al., '95a,b; Holz et al., '95; Moens et al., '96). In non-pancreatic cells, the signaling pathways are even less well defined (see Plisetskaya and Mommsen, '96). This study may not have properly optimized the GLP effects, and the role of IP_3 and Ca^{2+} needs to be better defined. The effects of PLC (Fig. 6), however, do support the possible involvement of this pathway in GLP signaling in the fish liver.

These studies are in agreement with those in mammals, which suggest that the GLU and GLP receptor signaling pathways are distinct. It is clear that GLU can function through both the cAMP and IP_3 system, but further studies are needed to define the appropriate pathway for GLP. The fish hepatocyte may, however, be an appropriate model for this study, as species differences between the responses to GLU, GLP, and EPI may assist in better understanding these pathways.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences & Engineering Research Council of Canada for their support of this work; EF was awarded a NSERC fellowship in support of her work in Ottawa, and TWM was awarded a NSERC travel award in support of studies in Ferrara. EF thanks Dr. M. Previati, Department of Anatomy, University of Ferrara, for assistance in the assay for PLC, and TWM thanks Dr. K.B. Storey, Biology, Carleton University of PKA assays.

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