Stimulation of Glycine Catabolism in Isolated Perfused Rat Liver by Calcium Mobilizing Hormones and in Isolated Rat Liver Mitochondria by Submicromolar Concentrations of Calcium*

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Glucagon stimulates flux through the glycine cleavage system (GCS) in isolated rat hepatocytes (Jois, M., Hall, B., Fewer, K., and Brosnan, J. T. (1989) J. Biol. Chem. 264, 3347–3351). In the present study, flux through GCS was measured in isolated rat liver perfused with 100 mM glucagon, 1 mM epinephrine, 1 mM norepinephrine, 10 mM phenylephrine, or 100 mM vasopressin. These hormones increased flux through GCS in perfused rat liver by 100–200% above the basal rate. The possibility that the stimulation of flux by adrenergic agonists and vasopressin is mediated by increases in cytoplasmic Ca²⁺ which in turn could regulate mitochondrial glycine catabolism was examined by measuring flux through GCS in isolated mitochondria in the presence of 0.04–2.88 μM free Ca²⁺. Flux through GCS in isolated mitochondria was exquisitely sensitive to free Ca²⁺ in the medium; half-maximal stimulation occurred at about 0.4 μM free Ca²⁺ and maximal stimulation (7-fold) was reached when the free Ca²⁺ in the medium was 1 μM. The Vₘₐₓ (nanomoles/mg protein/min) and Kₘ (millimolar) values for the flux through GCS in intact mitochondria were 0.67 ± 0.16 and 20.66 ± 4.82 in the presence of 1 mM ethylenebis(oxyethylenenitrilo]tetraacetate acid and 3.28 ± 0.76 and 10.98 ± 1.91 in presence of 0.5 μM free Ca²⁺, respectively. The results show that the flux through GCS is sensitive to concentrations of calcium which would be achieved in the cytoplasm of hepatocytes stimulated by calcium-mobilizing hormones.

The glycine cleavage enzyme system (GCS),¹ also known as glycine synthase (EC 2.1.2.10) represents a major pathway of catabolism of glycine in vertebrates (Yoshida and Kikuchi, 1970). GCS which consists of four enzyme proteins, present as an enzyme complex in the inner mitochondrial membrane, is most abundant in hepatic mitochondria and catalyses the following reaction:

\[
\text{Glycine} + \text{NAD}^+ + \text{tetrahydrofolate} \rightarrow \text{CO}_2 + \text{NH}_3 + 5\text{N}^\text{IV}\text{N}-\text{methylene tetrahydrofolate} + \text{NADH} + \text{H}^+ 
\]

The structure and mechanism of action of GCS have been studied in detail especially by Kikuchi and co-workers (Kikuchi, 1973). However, information is lacking on the physiological regulation of GCS activity. Previously proposed mechanisms of regulation of GCS activity include control by branched-chain α-keto acids (O'Brien, 1978; Kochi et al., 1986) and by the oxidation-reduction state of mitochondrial pyridine nucleotides (Hampson et al., 1983, 1984). We have recently shown that flux through GCS is regulated by glucagon and that the mechanism of regulation is independent of changes in the redox state of mitochondrial pyridine nucleotides (Jois et al., 1989). In the present study we show that epinephrine, norepinephrine, phenylephrine, and vasopressin also stimulate flux through GCS in isolated perfused rat liver. We also show that, in isolated mitochondria, flux through GCS is sensitive to submicromolar concentrations of free Ca²⁺.

**MATERIALS AND METHODS**

[¹^14C]Glycine was obtained from Du Pont-New England Nuclear. All hormones were obtained from Sigma. All other reagents were of analytical grade.

Livers of male Sprague-Dawley rats (150–250 g body weight) were perfused without recirculation as described previously (Sies, 1978). Perfusion consisted of Krebs-Henseleit buffer, 2.1 mM lactate, 0.3 mM pyruvate, and 0.3 mM [¹^14C]glycine. Hormones were infused (0.018 ml/min) into the perfusion system before the portal vein using a micro-pump (Harvard apparatus). Perfusion was collected every 5 min for 30 s under mineral oil. [¹^14C]CO₂ was released from 5-ml aliquots of perfusate samples by introducing the samples into 25-ml Erlenmeyer flasks containing 0.4 ml of 1 N HCl and fitted with rubber stoppers through which plastic center wells containing 0.4 ml of NCS tissue colubizer were suspended. [¹^14C]CO₂ was collected and counted as ¹⁵NCO₂ released and the specific activity of [¹^14C]glycine infused.

Mitochondria were isolated from livers obtained from male Sprague-Dawley rats (150–250 g body weight). The liver was homogenized in a medium containing 0.226 M mannitol, 0.076 M sucrose, 5 mM Hepes, and 1 mM EGTA (pH 7.2). The homogenate was centrifuged for 10 min at 2200 × g. The mitochondria were separated from the supernatant by centrifugation at 8200 × g. The pellet was washed once and resuspended in the homogenization medium and respun. The final mitochondrial pellet was resuspended in the same medium to give 30–40 mg of protein/ml. The mitochondrial protein was measured using a biuret procedure (Gornall et al., 1949) using bovine serum albumin as standard. An aliquot of the mitochondrial suspension was used to give a final concentration of 1 mg of protein/ml in the incubation flasks. Mitochondria were incubated for 15 min at 30°C under state 3 conditions in 25-ml Erlenmeyer flasks with constant agitation. The incubation medium consisted of 125 mM KCl, 10 mM Mops, 7 mM Tris base, 2.5 mM potassium phosphate, 2.5 mM magnesium chloride, 5 mM sodium chloride, 1 mM ADP, 5 mM [¹^14C]glycine, 1 mM EGTA, and 0–0.95 mM CaCl₂ to give free Ca²⁺ concentration ranging from 0 to 2.88 μM. Preparation of stock solutions of EGTA and EGTA + CaCl₂ are described previously (Denton et al., 1978). Free Ca²⁺ was calculated from CaCl₂:EGTA ratios using a computerized program (EQCAL, Biosoft, NJ) in which the equilibrium composition is determined by a free energy minimization procedure.
RESULTS AND DISCUSSION

We have previously shown that glucagon stimulates flux through GCS in isolated rat hepatocytes (Jois et al., 1989). Dibutryryl-cAMP was also equally effective in stimulating the flux suggesting that the effects of glucagon may be mediated by changes in hepatic cAMP levels. However, as many of the metabolic effects of glucagon can be mimicked by \( \alpha \)-adrenergic agonists via cAMP-independent mechanisms, the present experiments were carried out to examine the effects of \( \alpha \)-adrenergic agonists on flux through GCS in rat liver. We employed the isolated perfused rat liver so as to avoid any receptor damage caused by proteases used in the preparation of hepatocytes. Fig. 1 shows that 0.1 \( \mu \)M glucagon, 1 \( \mu \)M epinephrine, 1 \( \mu \)M norepinephrine, 10 \( \mu \)M phenylephrine, and 0.1 \( \mu \)M vasopressin all increased flux through GCS by 100–200% above basal rate in isolated perfused rat liver. In general the stimulatory effects were evident 10 min after hormone infusion and maximal stimulation was evident after about 20 min. \( \alpha \)-Adrenergic agonists and vasopressin are known to exert their effects in the liver by increasing the concentration of free Ca\(^{2+}\) in the cytoplasm (Williamson et al., 1985; Exton, 1985). In isolated hepatocytes, these hormones increase cytosolic free Ca\(^{2+}\) concentration from resting values of 0.1–0.2 \( \mu \)M to about 0.6 \( \mu \)M (Murphy et al., 1980; Blackmore et al., 1982; Charest et al., 1983; Thomas et al., 1984). Thus, the present experiments along with our previous work (Jois et al., 1989) suggest that both cAMP and Ca\(^{2+}\) may be important signals in hormonal stimulation of glycine catabolism in rat liver. However, the possibility that the effects of glucagon are also mediated by increase in cytoplasmic Ca\(^{2+}\) cannot be ruled out as both glucagon and cAMP have been shown to elevate cytoplasmic Ca\(^{2+}\) levels (Charest et al., 1983; Sistare et al., 1985).

Fig. 2 shows data from experiments in which rat liver mitochondria were incubated in the presence of varying concentrations of free Ca\(^{2+}\). Flux through GCS in rat liver mitochondrion was exquisitely sensitive to concentrations of free Ca\(^{2+}\) which would be expected to occur in the cytosol of hormonally stimulated hepatocytes. Half-maximal stimulation occurred around 0.4 \( \mu \)M whereas maximal stimulation (4-fold) occurred around 1 \( \mu \)M free Ca\(^{2+}\). The stimulation of flux through GCS by Ca\(^{2+}\) was also observed when mitochondria were incubated under state 4 conditions (in absence of ADP in the medium): the values for flux in absence and presence of 0.5 \( \mu \)M free Ca\(^{2+}\) were 0.05 ± 0.01 and 0.90 ± 0.26 nmol/mg protein/min, respectively (mean ± S.E., \( n = 8 \)). The stimulation of flux by Ca\(^{2+}\) was dependent on entry of Ca\(^{2+}\) into mitochondria as it was abolished by 1 \( \mu \)g/ml ruthenium red, an inhibitor of Ca\(^{2+}\) uptake by mitochondria (Fig. 3). Cysteamine (1 \( \text{mM} \)), a known inhibitor of GCS (Yudkoff et al., 1981) abolished \(^{14}\)CO\(_2\) production from [\( ^{1-}\text{H}]\)glycine both in presence and absence of Ca\(^{2+}\) (data not shown). The basal flux through GCS measured in the present study is significantly lower than values previously reported by us (Jois et al., 1989) as well as others (Yoshida and Kikuchi, 1970; Hampson et al., 1983). This discrepancy is due to failure to use EGTA to maintain low Ca\(^{2+}\) concentrations during mitochondrial isolation and incubation in the previous studies. In the absence of a Ca\(^{2+}\) chelating agent or specific precautions to prevent contamination, the incubation medium is likely to have a large contamination of free Ca\(^{2+}\), up to 20–30 \( \mu \)M (Campbell and Siddle, 1976). The results from the present experiment draw attention to the necessity of controlling the free Ca\(^{2+}\) in media when measuring flux through GCS.

The release of \(^{14}\)CO\(_2\) as a function of glycine concentration was examined in the presence (0.5 \( \mu \)M) and absence of free Ca\(^{2+}\) (Fig. 4). The \( V_{\text{max}} \) and \( K_a \) were 0.67 ± 0.16 and 20.66 ± 4.82 in absence of free Ca\(^{2+}\) (1 \( \text{mM} \) EGTA present) and 3.28 ± 0.76 and 10.98 ± 1.92 in presence of 0.5 \( \mu \)M free Ca\(^{2+}\), respectively (mean ± S.E., \( n = 6 \)). Thus a submicromolar concentration of free Ca\(^{2+}\) increased the \( V_{\text{max}} \) by 5-fold and decreased the \( K_a \) by 50%.

That glycine catabolism is stimulated by glucagon, catacholamines, and vasopressin is probably related to the fact that glycine is a glucogenic amino acid (Hetenyi et al., 1988) and that the gluconeogenic pathway from glycine involves the combined action of GCS and serine hydroxymethyl transferase such that two molecules of glycine produce one molecule each of serine and CO\(_2\). The serine can, then, be converted to...
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Fig. 2. Dose-response curve for effects of calcium on flux through GCS in isolated mitochondria. Isolated mitochondria were incubated under state 3 conditions with 5 mM [1-\(^{14}\)C]glycine and various concentrations of free Ca\(^{2+}\). Details of isolation of mitochondria, Ca-EGTA buffers, and measurement of flux through GCS are given under "Materials and Methods." Values are expressed as the mean ± S.E. of four separate experiments.

Fig. 3. Effect of ruthenium red on stimulation of flux through GCS by calcium. Mitochondria were incubated under state 3 conditions with or without 0.5 nM free Ca\(^{2+}\) and in presence or absence of 1 \(\mu\)g/ml ruthenium red. Other details are given under “Materials and Methods.” Values are presented as the mean ± S.E. of four separate experiments.

Fig. 4. Effect of 0.5 nM free Ca\(^{2+}\) on \(^{14}\)CO\(_2\) production from various concentrations of [1-\(^{14}\)C]glycine in isolated mitochondria. Isolated mitochondria were incubated under state 3 conditions with or without 0.66 \(\mu\)M free Ca\(^{2+}\) and in presence of various concentrations of [1-\(^{14}\)C]glycine. Other details are given under “Materials and Methods.” Values are expressed as the mean ± S.E. of six separate experiments.

Glucose, glucagon, catecholamines, and vasopressin have all been shown to increase hepatic glucose output by stimulating glycogenolysis and gluconeogenesis. Thus the physiological importance of the stimulation of flux through GCS by these three different classes of hormones probably relates to their stimulation of gluconeogenesis.

A number of mechanisms have been suggested whereby calcium acts as an intramitochondrial mediator of hormonal effects (Williamson et al., 1981; Denton and McCormack, 1985; and Halestrap, 1989). These include stimulation of calcium-sensitive enzymes, stimulation of respiration and an increase in matrix volume which, in turn, has been linked to stimulation of pyruvate carboxylation, citrulline synthesis, glutaminase, activity, etc. Further work is required to establish the precise mechanism by which Ca\(^{2+}\) stimulates flux through GCS in isolated liver mitochondria. However, the experiments reported in this paper clearly demonstrate the regulation of this enzyme in the perfused liver by calcium-mobilizing hormones. The experiments in isolated mitochondria clearly demonstrate that calcium itself is capable of mediating effects of these hormones.

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