Hepatic inositol release upon hormonal stimulation of perfused rat liver

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A sustained increase in the hepatic release of ^3H radioactivity was shown to occur upon hormonal stimulation of perfused rat liver 15–20 h after intraperitoneal injection of 100 μCi of *myo*-[2- ^3H]inositol. Hormone-released radioactive material was analysed by t.l.c. and was found to consist predominantly of [^3H]inositol, without further metabolites. Vasopressin (14 nM), phenylephrine (1.7 μM), angiotensin II (15 nM), glucagon (0.5 nM) and dibutyryl cyclic AMP (5 μM) exert maximal effects on hepatic inositol efflux after 10–15 min of stimulation. Omission of Ca^{2+} from the perfusion medium abolishes the hormone-dependent inositol release. LiCl (10 mM) does not significantly affect the basal release of [^3H]inositol, but suppresses vasopressin- and angiotensin-triggered inositol release. Inositol efflux induced by glucagon, dibutyryl cyclic AMP and phenylephrine, however, remains essentially unchanged by LiCl infusion. This establishes a further metabolic difference between these two groups of agonists in that stimuli that act through cyclic AMP produce a stimulated outflow of inositol, but apparently without a Li^+ -sensitive phosphatase being involved in the overall process.

INTRODUCTION

Various hormones and vasoactive pharmacological agonists have been described to cause intracellular hydrolysis of phosphatidylinositol 4,5-bisphosphate in liver (Creba *et al.*, 1983; Thomas *et al.*, 1984). Receptor-mediated production of inositol polyphosphates with subsequent degradation by different phosphatases (Storey *et al.*, 1984; Seyfred *et al.*, 1984) is a regulatory mechanism of intracellular Ca^{2+} mobilization. Here we investigate the release of free *myo*-inositol from perfused rat liver under steady-state conditions upon stimulation with hormones such as vasopressin and angiotensin II on the one hand and glucagon, dibutyryl cyclic AMP and phenylephrine on the other. Li^+ is known as an inhibitor of inositol-1-phosphatase (Hallcher & Sherman, 1980). Therefore the suppression of hormone-dependent inositol release by added LiCl was examined.

METHODS AND MATERIALS

Haemoglobin-free rat liver perfusion

Livers of male Wistar rats (170–240 g body wt.), fed on stock diet (Altromin, Lage, Germany), were perfused as described previously without recirculation (open system) at 37 °C (Sies, 1978). Additions were made by micro-pumps directly before the portal vein. Perfusate flow was constant throughout the individual experiments, at about 4 ml/min per g wet wt.

For anoxia, the gas mixture of O_2/CO_2 (19:1) was replaced by N_2/CO_2 (19:1).

Effluent-perfusate assays

O_2 concentration and pH in the effluent perfusate were continuously monitored with Clark-type and glass pH electrodes respectively. Lactate dehydrogenase activity and glucose concentration in the effluent perfusate were determined by assays as described in Bergmeyer (1983).

Measurement and identification of inositol

To investigate hormone-dependent inositol release, the animals were pretreated 15–20 h before the experiment by intraperitoneal injection with 100 μCi of *myo*-[2- ^3H]inositol in 0.9% NaCl (see Rhodes *et al.*, 1983). Released radioactivity was determined by adding 10.5 ml of liquid-scintillation fluid to 1 ml of perfusate and counted in a Kontron MR 300 liquid-scintillation counter for 5 min.

For identification of released radioactive material, 200 ml of effluent perfusate obtained after hormone stimulation of the liver was rapidly filtered through filter paper (Schleicher & Schüll, no. 597 $\frac{1}{2}$) and deproteinized with 8 ml of 2 M- HClO_4 . After centrifugation at 5000 g for 10 min, the supernatant was neutralized with 2 M-KOH/0.3 M-Mops buffer to pH 7.0, cooled to –70 °C to precipitate the KClO_4 and centrifuged again at room temperature. To decrease the concentration of salt, the supernatant was passed through 200 ml of a cation-exchanger gel, Amberlite IR-120, and subsequently through 200 ml of an anion-exchanger gel, Amberlite IRA-400. Repeating this procedure once with 20 ml each of cation and anion exchangers decreased the salt concentration to 3×10^{-4} of the initial value, as measured by conductivity. To decrease the volume of the effluent perfusate, evaporation was performed with a Büchi Rota-Vapor R 1 at 40 °C to a final volume of 50 μl . Recovery of hormone-released radioactivity by this method amounted to 40–50%, similar to the results obtained with a *myo*-[2- ^3H]inositol standard of comparable radioactivity and analysed by the same procedure.

For chromatographic identification, 5 or 10 μl of volume-concentrated desalted perfusate was applied to silica-gel plates. T.l.c. was carried out in propan-1-ol/ethyl acetate/water/25% (v/v) NH_3 (5:1:3:1, by vol.) as solvent. Dried t.l.c. plates were analysed for radioactivity with an Isomess t.l.c. radio-scanner.

Determination of intracellular inositol phosphates

Hepatic inositol phosphate contents were determined as described previously (Graf *et al.*, 1987) by anion-exchange chromatography with Dowex 1X8 columns (0.5 cm × 2 cm) by the method described by Downes & Michell (1981) and Berridge *et al.* (1983), with elution of inositol phosphates as described by Batty *et al.* (1985).

Materials

[Lysine]vasopressin (grade I-S, oxytocin-free), angiotensin II, glucagon and phenylephrine were obtained from Sigma (Munich, Germany). Prazosin was a gift from Pfizer (Karlsruhe, Germany). *myo*-[2-³H]inositol (16.3 Ci/mmol) and *D*-*myo*-[2-³H]inositol 1,4,5-trisphosphate (1 Ci/mmol) were from Amersham Buchler (Braunschweig, Germany). *D*-[1-¹⁴C]Glucose (6.5 mCi/mmol) was from New England Nuclear, Dreieich, Germany. Liquid-scintillation fluid (Quickszint 212) was provided by Zinsser Analytic (Frankfurt, Germany). Amberlite and Dowex gels were supplied by Serva (Heidelberg, Germany). Dibutyryl 3',5'-cyclic AMP and biochemicals were from Boehringer (Mannheim, Germany). All other chemicals and the silica-gel plates (60 F₂₅₄; 20 cm × 20 cm) were obtained from Merck (Darmstadt, Germany).

RESULTS AND DISCUSSION

Hepatic inositol release upon hormonal stimulation with vasopressin

The fate of radioactive *myo*-inositol after intraperitoneal injection into mature male rats has been investigated by Lewin *et al.* (1976): 5 h after the injection, the liver was found to accumulate 10% of the administered dose, predominantly present in the lipid fraction. In experiments with isolated hepatocytes (Prpic *et al.*, 1982a), 95% of labelled phospholipids were present as phosphatidylinositol and 4% as phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. At 15–20 h after injection of *myo*-[2-³H]inositol, we found in the haemoglobin-free liver an accumulation of $4.9 \pm 0.5\%$ ($n = 4$) of the administered dose.

By perfusing the liver with Krebs–Henseleit (1932) medium, we observed a constant release of radioactive *myo*-inositol into the perfusion medium 15–20 h after intraperitoneal injection of *myo*-[2-³H]inositol (Fig. 1a). This basal release reaches a steady state at 30–40 min of perfusion time and amounts to about 220 d.p.m./min per ml of effluent perfusate. This basal release would correspond to 24 fmol of inositol equivalents/min per g of liver if there were no dilution of label by endogenous inositol pools; therefore, this value represents a lower limit. The experiments shown below are presented for the steady-state period between 50 and 120 min of perfusion time, with the hormone infusion usually beginning at 60 min.

Infusion of vasopressin (14 nM) causes a sustained increase in the release of radioactivity into the perfusate, with the maximal effect reached after 10 min of stimulation. This concentration of agonist is supramaximal with respect to the physiological response (Creba *et al.*, 1983; Graf *et al.*, 1987), but slightly sub-maximal with respect to receptor occupancy (Creba *et al.*, 1983); a higher dose than 14 nM was not employed in this study.

Fig. 1. Vasopressin-dependent inositol release and its inhibition by LiCl in perfused rat liver

(a) Time course of inositol release upon infusion of vasopressin (●) and of vasopressin plus LiCl (○) in the isolated perfused rat liver. Inositol was measured as ³H released (d.p.m.) per ml of effluent perfusate. For identification of radioactive material see the text. (b) Glucose release upon additions of vasopressin (■) and vasopressin plus LiCl (□). Each point represents the mean ± S.E.M. for at least three different experiments. (c) Lactate dehydrogenase (LDH) activity in the effluent perfusate. Each point represents means ± S.E.M. from four experiments with vasopressin infusion. LiCl infusion did not cause a significant increase in lactate dehydrogenase release from perfused rat liver. O₂ uptake, depicted in (d), represents one experiment typical of three; there was no difference in the O₂ uptake in the presence of LiCl.

During a stimulation period of 30 min (from 60 to 90 min) the sum of the mobilized radioactivity amounted to about 0.2% of the injected dose. To exclude the possibility of severe cellular damage, lactate dehydrogenase release into the effluent perfusate was monitored for the experimental time period. In the experiments presented below, the lactate dehydrogenase activity in the effluent perfusate increased from 0.5 to about 5 units/l

from 40 to 90 min (results not shown), corresponding to maximally 20 munits/min per g of liver (Fig. 1c), which is approx. 0.01% of the total hepatic lactate dehydrogenase activity/min. Further, there was no increase in lactate dehydrogenase release upon addition of hormone. This indicates that leakage of inositol radioactivity caused by cellular damage is not responsible for the observed effects. The metabolic responses to hormone addition, such as glucose release and O_2 uptake, were also measured (Figs. 1b and 1d).

Effect of LiCl on vasopressin-dependent inositol release

Vasopressin-mediated [3H]inositol efflux is completely suppressed by infusion of LiCl (10 mM) after 14 min of stimulation (Fig. 1a). Also there is a slight decrease of glucose release with LiCl (Fig. 1b). Hormone-dependent effects are not restored after cessation of the LiCl infusion after 6 min (at 80 min perfusion time). Infusion of 10 mM-LiCl 10 min before the addition of vasopressin (14 nM) prevents the increase in the release of [3H]inositol (Fig. 2a). Glucose release begins to decline 2 min earlier with LiCl added (Fig. 2b). The time course (Fig. 1a) of suppression of [3H]inositol efflux towards the initial

Fig. 2. Suppression of vasopressin-induced efflux of [3H]inositol in the presence of LiCl

(a) [3H]Inositol efflux with vasopressin (●) and with LiCl plus vasopressin (○). (b) Glucose release upon additions of vasopressin (■) and of LiCl plus vasopressin (□). LiCl was infused from 50 to 70 min, and the hormone was added at 60 min. Where indicated, livers were freeze-clamped for measurements of intracellular inositol polyphosphates as shown in Fig. 3.

value before LiCl addition (about 10 min) may be governed either by slow Li^+ entry or slow inositol-1-phosphatase inhibition, or else by slow efflux of inositol already in the free inositol pool.

To examine intracellular inositol polyphosphates in vasopressin-stimulated liver after LiCl addition from 50 to 70 min, the livers were freeze-clamped after 20 min of hormonal stimulation with vasopressin at time point 80 min as indicated in Fig. 2(a), and the samples were further processed as described in the Methods and materials section. As revealed in Fig. 3 for an experiment representative of four, LiCl infusion before addition of vasopressin leads to an accumulation of [3H]inositol 1-phosphate up to 3-fold above the control value. This corresponds to an increase in intracellular [3H]inositol monophosphate from 97 to 257 fmol/g of liver. As the additional vasopressin-triggered release of [3H]inositol amounts to about 450 fmol of [3H]inositol/g of liver after 20 min of hormonal stimulation, it can be calculated that the increase in [3H]inositol monophosphate accounts for about 40% of the expected vasopressin-dependent [3H]inositol release, assuming a complete inhibition of inositol-1-phosphatase by addition of LiCl.

Dependence of extracellular Ca^{2+} on hepatic inositol release

Omission of Ca^{2+} from the perfusion medium characteristically alters the shape of the time course and the extent of vasopressin-induced [3H]inositol release (Fig. 4). In the experiment depicted, representative of five, the liver was perfused with Ca^{2+} -free medium from 30 to 74 min, which itself did not alter the basic release of [3H]inositol significantly (results not shown). Vasopressin infusion at saturating concentration (13 nM) at 60 min

Fig. 3. Elution patterns of inositol phosphates from vasopressin-stimulated livers in the presence and absence of LiCl

Perfusions were performed with (○) and without (●) LiCl infusion before the hormone addition as described in Fig. 2. After 20 min of stimulation with vasopressin, livers were freeze-clamped (see arrow in Fig. 2). Samples (1–2 g) were further processed and eluted from Dowex 1X8 columns. Patterns are representative for four perfusions. Data are expressed as d.p.m./150 mg of liver protein. Abbreviations: IP₁, inositol monophosphate (1- or 4-); IP₂, inositol 1,4-bisphosphate; IP₃, inositol trisphosphate (1,3,4- or 1,4,5-); IP₄, inositol 1,3,4,5-tetrakisphosphate.

Fig. 4. Dependence of extracellular Ca^{2+} concentration on hepatic inositol release

The liver was perfused with Ca^{2+} -free medium from 30 to 74 min. Results are representative of five different perfusions.

evokes a transient response, as opposed to the sustained response with physiological free Ca^{2+} concentration shown in Fig. 1(a). Infusion of Ca^{2+} (1.25 mM) at 74 min restores the vasopressin-dependent inositol release. These results suggest a requirement for extracellular Ca^{2+} in hepatic inositol liberation.

Identification of radioactive material released upon hormone stimulation of the liver

Effluent perfusate obtained after vasopressin stimulation of the liver was analysed to identify the released radioactive material as described in the Methods and materials section. Hormone-mobilized radioactive material co-chromatographs with *myo*-[2- ^3H]inositol standard, as shown in Fig. 5. The chromatographic system allows a clear separation of *myo*-[^3H]inositol from other non-polar carbohydrates, e.g. [^{14}C]glucose, whose migration distance is 47 mm in this chromatographic system (results not shown). To exclude the possibility of liberation of [^3H]inositol-containing anionic metabolites from perfused rat liver, the effluent perfusate after hormone stimulation was filtered and eluted from Dowex 1X8 columns as described for the intracellular inositol polyphosphate measurement; no phosphorylated water-soluble inositol metabolites were detected. [^3H]Inositol 1,4,5-trisphosphate remains at the origin. In conclusion, we suggest that the radioactive material released upon hormonal stimulation predominantly consists of [^3H]inositol.

Fig. 5. Comparison of thin-layer chromatograms of *myo*-[2- ^3H]inositol standard and vasopressin-stimulated effluent perfusate

Effluent perfusate was collected during maximal stimulation of hepatic inositol efflux with vasopressin. The *myo*-[2- ^3H]inositol standard was dissolved in Krebs–Henseleit buffer. Both preparations were processed as described in the Methods and materials section. Results for subsequent separation by t.l.c. are shown for the [^3H]inositol standard (top) and for the hormone-liberated radioactivity (bottom).

Effects of different agonists on hepatic inositol efflux

We also investigated the effects of other hormones and agonists on hepatic inositol release and its inhibition by LiCl. Glucagon (0.5 nM), dibutyryl 3',5'-cyclic AMP (5 μM), phenylephrine (1.7 μM) and angiotensin II (15 nM) also induce a significant and sustained increase in the release of labelled inositol (Table 1). Whereas angiotensin-II- and vasopressin-triggered inositol efflux is suppressed by LiCl infusion, there is no significant change in the release of radioactive inositol evoked by those agonists related to the adenylate cyclase pathway, e.g. glucagon (Fig. 6) or dibutyryl cyclic AMP. Phenylephrine-induced inositol release is completely inhibited by infusion of the α -receptor antagonist prazosin at 3.2 μM concentration (results not shown). We also examined anoxia, which effects a moderate increase in [^3H]inositol released from perfused rat liver.

Concluding remarks

In the present investigation we describe the effect of hormone addition on hepatic inositol release by using labelled inositol in the experimental model of perfused rat liver. From an intraperitoneally injected dose of 6 nmol of *myo*-[2- ^3H]inositol, the liver accumulates approx. 300 pmol. The hormone-induced increase in the release of labelled inositol above the basal rate amounts to 0.2 pmol/min. This accounts for about 6 pmol during 30 min of stimulation, which is about 2% of the

Table 1. Effects of hormones and anoxia on hepatic inositol release

Conditions were as in Fig. 1. Additions were made from 60 to 90 min of perfusion. LiCl (10 mM) was added from 74 to 90 min. Data are expressed as fold increase in effluent-perfusate radioactivity. Control values at 60 min were measured immediately before the addition of agonist or N₂ (anoxia). Control values were set equal to 1.00 and were about 220 d.p.m./ml of effluent perfusate for [³H]inositol, but differed slightly between the different experimental groups. There were stable steady states over the experimental period, so that there was no increase in released ³H radioactivity between 60 and 90 min when no further additions were made. Data are given as means $\pm$ S.E.M., for the numbers of experiments in parentheses.

[LiCl]...	Increase (fold) in ³ H radioactivity from 60 to 90 min	
	None	10 mM
Vasopressin (14 nM)	1.84 $\pm$ 0.11 (4)	1.00 $\pm$ 0.15 (4)
Angiotensin II (15 nM)	1.97 $\pm$ 0.22 (3)	1.04 $\pm$ 0.13 (3)
Glucagon (0.5 nM)	1.59 $\pm$ 0.23 (4)	1.51 $\pm$ 0.16 (3)
Dibutyl cyclic AMP (5 μ M)	2.42 $\pm$ 0.87 (3)	2.27 $\pm$ 0.63 (3)
Phenylephrine (1.7 μ M)	1.35 $\pm$ 0.16 (3)	1.41 $\pm$ 0.28 (3)
Anoxia	1.61 $\pm$ 0.43 (3)	1.31 $\pm$ 0.27 (5)

Fig. 6. Glucagon-dependent inositol release

(a) Inositol release upon additions of glucagon (●) and glucagon plus LiCl (○). (b) Glucose release upon additions of glucagon (■) and glucagon plus LiCl (□). Conditions were as in Fig. 1. LiCl was infused from 74 to 90 min of perfusion.

radioactivity incorporated into the liver (the total amount of released inositol may be higher, because of dilution by unlabelled endogenous inositol).

In agreement with the results of Prpic *et al.* (1982b), who demonstrated the cessation of the hormone-induced increase in [³H]inositol in isolated rat hepatocytes by lowering extracellular Ca²⁺ concentration, Ca²⁺ omission

from the perfusion medium abolishes the hormone-induced effects. Thus it can be concluded that extracellular Ca²⁺ is essential for inositol release from the hepatocyte. This effect is likely to be due to the intracellular Ca²⁺ requirement for phosphoinositidase C characterized by Creba *et al.* (1983). Another explanation could be an effect of extracellular Ca²⁺ itself on transmembrane inositol flux.

A further aim of this study was to examine the influence of LiCl on inositol release from the liver. Whereas LiCl does not alter the basal release of radioactivity attributable to inositol as observed without hormone addition (Fig. 2a), the inositol release induced by vasopressin or angiotensin II is completely suppressed by LiCl infusion (Fig. 1a, Table 1). The effects of other agonists which cause an inositol efflux comparable with that produced by vasopressin, i.e. glucagon, dibutyl 3',5'-cyclic AMP and phenylephrine, are not influenced by LiCl addition. These results suggest the existence of different intracellular hormone-sensitive inositol pools, which can be distinguished by their sensitivity to LiCl. As Li⁺ blocks intracellular breakdown of inositol phosphates resulting from agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate, we therefore suggest one source of mobilized inositol to originate from a Li⁺-sensitive dephosphorylation of inositol polyphosphates. Another pool of inositol, predominantly observed under basal conditions and upon hormonal stimulation with agonists such as glucagon and dibutyl cyclic AMP, is Li⁺-insensitive. Although vasopressin and glucagon mobilize hepatic Ca²⁺ to a similar extent (Kraus-Friedmann, 1986), the mechanism responsible for glucagon-induced Ca²⁺ release still remains to be elucidated. Experiments by Williamson & Hansen (1987) and Poggioli *et al.* (1986) showed a negligible increase in inositol 1,4,5-trisphosphate after incubation of hepatocytes with glucagon at 10 and 100 nM, although inositol 1,3,4,5-tetrakisphosphate showed an increase of about 40%. Thus it is still under debate as to what extent glucagon affects intracellular polyphosphate production and in which way cyclic AMP-dependent processes are involved [see also the previous work of Billah & Michell (1979) and Kirk *et al.* (1981)]. A possible explanation for

the lack of sensitivity of glucagon-induced inositol release to Li^+ could be differences in the degree of inhibition of inositol-1-phosphatase and inositol-4-phosphatase in liver by Li^+ . This is still a matter of controversy. Michell (1986) found inositol 4-phosphate degradation to be Li^+ -insensitive (see also Fig. 1 in Berridge, 1987), whereas more recent work by Delvaux *et al.* (1987) revealed Li^+ -sensitivity for both pathways, via inositol 1-phosphate and inositol 4-phosphate, in liver. Further, Wakelam *et al.* (1986) described a second class of hepatic glucagon receptors whose actions are mediated through inositol lipid hydrolysis, and it is now clear (Ackermann *et al.*, 1987) that inositol-1-phosphatase hydrolyses a variety of different *myo*-inositol monophosphates in a Li^+ -inhibitable manner.

In conclusion, we suggest that insensitivity of glucagon-dependent inositol release to LiCl reflects different pathways in inositol polyphosphate catabolism as compared with vasopressin. Although further research is required into the intracellular events that precede extracellular inositol release in liver, we here present an experimental tool with which to monitor hormone-induced generation of inositol polyphosphates by extracellular measurement of free inositol.

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