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Activation of inositol phosphate formation by circulating noradrenaline but not by sympathetic nerve stimulation with a similar increase of glucose release in perfused rat liver

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In the isolated rat liver perfused *in situ*, stimulation of the nerve bundles around the hepatic artery and portal vein caused an increase of glucose and lactate output and a reduction of perfusion flow. These changes could be inhibited completely by α-receptor blockers. The possible involvement of inositol phosphates in the intracellular signal transmission was studied.

1. In cell-suspension experiments, which were performed as a positive control, noradrenaline caused an increase in glucose output and, in the presence of 10 mM LiCl, a dose-dependent and time-dependent increase of inositol mono, bis and trisphosphate.
2. In the perfused rat liver 1 μM noradrenaline caused an increase of glucose and lactate output and in the presence of 10 mM LiCl a time-dependent increase of inositol mono, bis and trisphosphate that was comparable to that observed in cell suspensions.
3. In the perfused rat liver stimulation of the nerve bundles around the portal vein and hepatic artery caused a similar increase in glucose and lactate output to that produced by noradrenaline, but in the presence of 10 mM LiCl there was a smaller increase of inositol monophosphate and no increase of inositol bis and trisphosphate.

These findings are in line with the proposal that circulating noradrenaline reaches every hepatocyte, causing a clear overall increase of inositol phosphate formation and thus calcium release from the endoplasmic reticulum, while the hepatic nerves reach only a few cells causing there a small local change of inositol phosphate metabolism and thence a propagation of the signal via gap junctions.

Electrical stimulation of the nerve bundles around the hepatic artery and portal vein in the isolated rat liver perfused *in situ* (review [1, 2]) increased glucose and lactate output, decreased perfusion flow [3, 4] and caused an overflow of noradrenaline into the hepatic vein [4, 5]. All nerve effects were dependent on the presence of Ca^{2+} in the perfusion medium [3, 5, 6] and were inhibited by general α-adrenergic antagonists like phentolamine [3, 5] and more specifically by the α_1-blocker prazosine [4, 7]. They were not accompanied by an increase of intracellular cAMP concentration [6] and could be mimicked in a first approximation by infusion of noradrenaline [3]. These findings showed that the metabolic and hemodynamic nerve effects are mediated via an α-adrenergic mechanism.

Inositol 1,4,5-trisphosphate is generally implicated as an internal signal to mobilize calcium from intracellular stores (review [8, 9]). It is regarded as the second messenger for the increase of cytosolic calcium as the third messenger in the intracellular signal transmission after stimulation of α-adrenergic receptors (review [10]). It has been shown in liver cell suspensions that the α-agonists phenylephrine [11], adrenaline [12–14], noradrenaline [15] and other calcium-mobilizing signals, such as angiotensin II [13, 15, 16], vasopressin [11–15, 17, 18] and ATP [13, 19], caused an increase of Ins(1,4,5)P_3 that coincided with the increase of intracellular Ca^{2+} concentrations [17] and preceeded the activation of glycogen phosphorylase [16, 17]. In the presence of LiCl, which inhibits the dephosphorylation of Ins1P and InsP_2 [20] and thereby slows down the degradation of all inositol phosphates, a sustained accumulation of the inositol phosphates could be observed after administration of the α-agonists.

It was the object of the present investigation to study the possible involvement of inositol phosphates as intracellular messengers in the metabolic actions of sympathetic hepatic nerve stimulation. It was found in the perfused rat liver that nerve stimulation and noradrenaline infusion caused a similar increase in glucose release but had different effects on inositol phosphate metabolism. Noradrenaline clearly increased InsP_1, InsP_2 and InsP_3, while nerve stimulation elevated InsP_1 only slightly and had almost no detectable effect on InsP_2 and InsP_3.

MATERIALS AND METHODS

Materials

All chemicals were of reagent grade and from commercial sources. Bovine serum albumin, lactate dehydrogenase

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Abbreviations. Ins, inositol; Ins1P_1, inositol 1-phosphate; Ins1,4P_2, inositol 1,4-bisphosphate; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate.

Enzymes. Collagenase (EC 3.2.24.3); glucose dehydrogenase (EC 1.1.1.47); glutamate pyruvate transaminase (EC 2.6.1.2); lactate dehydrogenase (EC 1.1.1.27).

and glutamic-pyruvic transaminase were purchased from Boehringer (D-6800 Mannheim), collagenase for liver cell preparation from Biochrom (D-1000 Berlin). Dowex X8, mesh 200–400, anion-exchange resin and noradrenaline were obtained from Serva (D-6900 Heidelberg). The Merck Glucose System, for glucose determination using the glucose dehydrogenase reaction, was from Merck (D-6100 Darmstadt), *myo*-[2-^{3}H]inositol from Amersham Buchler (D-3300 Braunschweig), *myo*-[2-^{3}H]inositol 1-phosphate, *myo*-[2-^{3}H]inositol 1,4-bisphosphate and *myo*-[2-^{3}H]inositol 1,4,5-trisphosphate from NEN radiochemicals (D-1000 Berlin). Hydroluma scintillation liquid was supplied by Baker (Deventer, The Netherlands).

Animals

Male Wistar rats (180–200 g, Winkelmann, D-4791 Borchen) were kept on a 12-h day/night rhythm, 7 a.m. to 7 p.m. light, with free access to water and food (standard rat diet, Ssniff, D-4770 Soest). All experiments were started between 9 a.m. and 10 a.m.

Cell-suspension experiments

Liver cells were prepared as described previously [21] basically according to Berry and Friend [22]. Liver cells (100 mg/ml) were suspended in a Krebs Henseleit bicarbonate buffer pH 7.4, which was equilibrated with 5% CO_2 and 95% O_2 and contained 5 mM glucose, 2 mM lactate, 0.2 mM pyruvate and 2% bovine serum albumin. The cell suspension was kept under the same gas mixture throughout the entire experiment. After addition of 10 μCi/ml *myo*-[2-^{3}H]inositol (0.64 μmol/l, 15.6 mCi/μmol) this cell suspension was incubated at 37 °C for 90 min in order to label the phosphoinositides. The cells were washed twice at room temperature in the above buffer containing 10 mM LiCl to inhibit the degradation of inositol phosphates [20] and finally suspended in this buffer. After 10 min preincubation either 1 μM or 10 μM noradrenaline were added and 0, 2, 5 and 10 min after addition of noradrenaline 0.5-ml samples were taken from the suspension. The samples were centrifuged immediately for 2 s in an Eppendorf microfuge to sediment the cells, supernatants were stored on ice for later glucose and lactate determination. The pellets were resuspended in 750 μl 10% trichloroacetic acid, left on ice for 30 min and then centrifuged in the Eppendorf microfuge for 15 min. 700 μl supernatant were extracted four times with 3 ml diethyl ether, the aqueous phase was freeze-dried and redissolved in 200 μl bidistilled H_2O. Inositol-containing water-soluble compounds were separated on Dowex formate columns (see below).

Perfusion experiments

Rats were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body mass). The liver was perfused *in situ* via the portal vein as described previously [3]. In order to label phosphoinositides the liver was perfused for 60 min in a recirculating mode with about 60–70 ml Krebs Henseleit buffer pH 7.4 containing 5 mM glucose, 2 mM lactate, 0.2 mM pyruvate, 30% washed bovine erythrocytes and 80 μCi *myo*-[2-^{3}H]inositol (0.075–0.085 μmol/l; 15.6 mCi/μmol). The medium was equilibrated with 95% O_2 and 5% CO_2. Subsequently the erythrocyte-containing medium and the majority of the unincorporated tracer were removed from the liver during a 30-min non-recirculating perfusion with Krebs Henseleit buffer pH 7.4 containing 5 mM glucose, 2 mM lactate and 0.2 mM pyruvate.

The perfusion was then continued with the same buffer that contained additionally 10 mM LiCl. After 10 min either noradrenaline was infused to a final concentration of 1 μM or the hepatic nerves around the hepatic artery and portal vein were stimulated with rectangular pulses (20 Hz, 2 ms) using a bipolar platinum electrode [3]. The perfusate was collected with a fraction collector at 1-min intervals and cooled on ice. 2 min before and 2 min, 5 min and 10 min after onset of the stimulus liver samples of 300–600 mg were taken with tongs cooled in liquid nitrogen and stored at −20 °C. The resulting leakage was tightened with wire clips.

Liver samples were prepared for further analysis at 0 to 4 °C. They were thawed in 10% trichloroacetic acid (1 g liver tissue/20 ml trichloroacetic acid) and homogenized with an Ultraturrax (Jahnke and Kunkel, D-7813 Staufen) for 60 s. After standing on ice for another 30 min the samples were centrifuged at 3000 × *g* for 20 min. The supernatants were extracted four times with an equal volume of diethyl ether, the ether phases were discarded, the final aqueous phase was lyophilized and redissolved in 1/20 original volume.

Separation of inositol phosphates

Inositol phosphates were separated using a modification of a method described by Downes and Michell [23] (cf. [17]). Dowex formate was prepared according to Cooper [24]. 100 μl concentrated sample from the cell suspension or perfusion experiments (see above) were applied to Dowex-formate columns and eluted as described in the legend to Fig. 1.

Metabolite assays

Glucose was determined using a commercial test kit (Merck Glucose System) based on the glucose dehydrogenase reaction [25]. Lactate was measured in a combined optical test using lactate dehydrogenase and glutamic pyruvic transaminase in a glutamate buffer pH 9.5 [26].

RESULTS

The involvement of inositol phosphates as second messenger in the hormonal regulation of liver metabolism has been studied so far only in liver cell suspensions but not in perfused livers [11–19]. Since the nervous regulation of metabolism can be studied in perfused livers only, the techniques used in the study of cell suspensions had to be adapted to the investigation of the intact perfused organ. First, a method to prelabel the phosphoinositides of the whole liver was developed; second, the usual column-chromatographic separation of labelled inositol phosphates [23] had to be modified to improve the separation of inositol phosphates from whole liver homogenates.

Labelling of phosphoinositides in the perfused liver

In a recirculating perfusion 80 μCi *myo*-[2-^{3}H]inositol were offered per 5.5–8 g liver, i.e. 10–15 μCi/g; the radioactive concentration in the recirculating medium was about 1 μCi/ml, the molar concentration about 0.08 μmol/l. During a 60-min recirculating perfusion, which was followed by a 30-min non-recirculating perfusion to wash out the bulk part of unincorporated tracer, about 1.7–2.6 μCi/g wet mass were

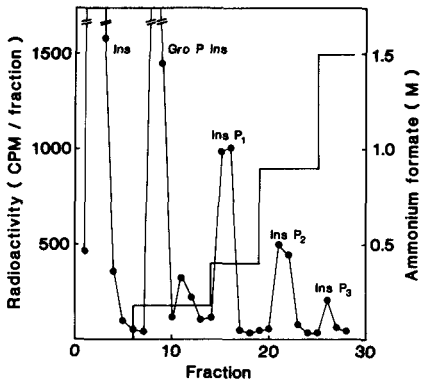

Fig. 1. *Separation of inositol phosphates on Dowex formate columns.* The elution profile of a single representative perfusion experiment is shown. 100 µl concentrated sample, prepared as described in Materials and Methods, were applied to a column containing 2 g moist resin of Dowex formate X8 (200–400 mesh, 5 mm inner diameter; height about 10 cm; volume about 2 ml). The column was eluted with a stepwise gradient of 5 ml bidistilled water, 8 ml 0.18 M ammonium formate, 5 ml 0.4 M ammonium formate in 0.1 M formic acid, 6 ml 0.9 M ammonium formate in 0.1 M formic acid, and 4 ml 1.5 M ammonium formate in 0.1 M formic acid. Fractions of 1 ml were collected, 750 µl of which were counted in 10 ml Hydroluma. The peaks were assigned definitely using authentic radiolabelled standards for Ins, Ins1P_1, Ins1,4P_2 and Ins(1,4,5)P_3, and tentatively for Gro*P*Ins (glycerophosphoinositol) by analogy to similar separations [17]

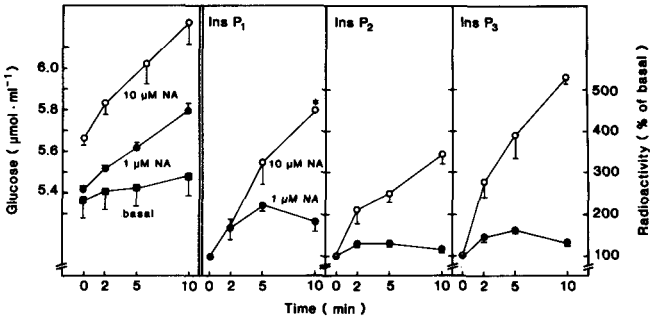

Fig. 2. *Increase of glucose output and inositol phosphate formation by noradrenaline in liver cell suspensions.* Liver cells (100 mg/ml) were labelled with *myo*-[2-^{3}H]inositol for 90 min. Unbound tracer was removed and cells supended in a Krebs Henseleit buffer containing 5 mM glucose, 2 mM lactate, 0.2 mM pyruvate, 2% bovine serum albumin and 10 mM LiCl. After 10 min noradrenaline was added to a final concentration of either 1 µM or 10 µM. At times 0, 2, 5 and 10 min after addition of the hormone, aliquots of the cell suspension were removed and centrifuged for 2 s. Glucose concentration was determined in the supernatants, the pellets were processed as described in Materials and Methods and the relative concentration of the inositol phosphates was determined as shown in Fig. 1. Values are means ± SEM for three experiments (* two experiments)

taken up by the liver, of which about 53% were water-soluble, the remaining 47% were bound to the particulate fraction (a 10000 × *g* sediment). The latter could be solubilized completely with a 10 mM Tris/HCl buffer pH 7.4 containing 2% Triton X-100. The majority of the water-soluble radioactivity did not bind to Dowex formate; it was eluted with the *myo*-inositol fraction and thus assumed to be *myo*-inositol that had been taken up and trapped by the liver but was not yet incorporated into the phosphoinositides.

This prelabelling of the phosphoinositides by a 60-min recirculating perfusion resulted in much higher degree of incorporation of radioactive tracer than the 20 h *in vivo* labelling carried out as reported previously [27] (single experiment, data not shown). However, it did not reach the extent of labelling possible in hepatocyte suspensions with a tenfold higher supply of label. Here about 100 µCi *myo*-[2-^{3}H]inositol were offered/g wet mass; the radioactive concentration of the tracer was about 10 µCi/ml, the molar concentration about 0.65 µmol/l. Under these conditions about 5 µCi/g wet mass were retained by the hepatocytes. This is two to three times more than in the perfused rat liver. 78% of the tracer was bound to the particulate fraction.

Separation of inositol phosphates

In order to get a good separation of trichloroacetic-acid-soluble inositol phosphates from liver tissue homogenates by chromatography on Dowex formate columns, as developed by Downes and Michell [23], the ammonium formate/formic acid step gradients for elution had to be modified (Fig. 1). The separation was controlled using authentic standards.

Stimulation of glucose output and inositol phosphate metabolism in hepatocyte suspensions by noradrenaline

In order to validate the analytical methods the effect of α-receptor stimulation was studied first in liver cell suspensions as previously reported by Thomas et al. [17]. However, instead of 20 nM vasopressin, used in the previous study, 1 µM and 10 µM of the α-agonist noradrenaline were applied. Noradrenaline caused a time-dependent and dose-dependent increase of InsP_1, InsP_2 and InsP_3 (Fig. 2). The changes were comparable to those described previously for 10 µM noradrenaline [15], which increased InsP_3 within 5 min to around 250% basal level under conditions similar to those used here, and for 10 µM adrenaline in the presence of 10 µM of the β-blocker propranolol [12], which increased InsP_1, InsP_2 and InsP_3 to about 112%, 196% and 261%, respectively, within 30 s. These findings show that the modified methods were reliable. Glucose output from the liver cells was increased to about 360% by 1 µM noradrenaline and to about 580% by 10 µM noradrenaline (Fig. 2).

Stimulation of glucose output and inositol phosphate metabolism in perfused liver by noradrenaline and sympathetic nerve stimulation

Infusion of 1 µM noradrenaline led to a large and sustained increase of all three inositol phosphates (Fig. 3). The extent was comparable to that observed in cell suspensions with the same concentration of noradrenaline; however, the increase of inositol phosphates in cell suspension was faster than in the perfused liver and had already started to decrease again after 5 min. This is most likely due to the fact that the noradrenaline concentration in the cell suspension decreased as a result of degradation whereas it was continously offered in the perfusion. The catecholamine also caused an increase of glucose output (area under the curve, means ± SEM) of 10.0 ± 1.4 µmol g liver^{-1} (Fig. 3) and lactate output (not shown) of 4.9 ± 0.5 µmol g liver^{-1} each and a decrease of flow of 5.6 ± 0.9 ml g^{-1} (not shown).

Nerve stimulation increased only InsP_1, and not InsP_2 or InsP_3 (Fig. 3). The InsP_1 increase was statistically significant only after 5 min of stimulation and started to decrease despite continued stimulation. InsP_2 showed a tendency to increase towards the fifth minute and to decrease again later. Although nerve stimulation had only a very weak effect on the formation

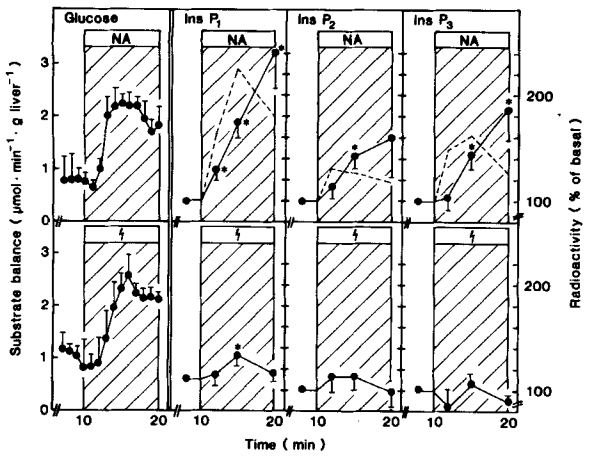

Fig. 3. *Increase of glucose output and inositol phosphate formation by noradrenaline and nerve stimulation in the perfused rat liver.* Livers were labelled with *myo*-[2-^{3}H]inositol during a 60-min recirculating perfusion followed by 30 min open perfusion to remove unincorporated tracer. Livers were then perfused with a Krebs Henseleit buffer containing 5 mM glucose, 2 mM lactate, 0.2 mM pyruvate and 10 mM LiCl. 10 min after the start of the experiment either noradrenaline was infused to a final concentration of 1 µM or nerve bundles around the hepatic artery and portal vein were stimulated electrically with a frequency of 20 Hz and a pulse width of 2 ms for 10 min. Substrate balance is given by [concentration in hepatic vein minus concentration in portal vein (µmol ml^{-1})] × flow (ml min^{-1} g liver^{-1}). Liver samples were taken 2 min before and 2 min, 5 min and 10 min after the onset of the stimulus and prepared as described in Materials and Methods. The relative concentration of inositol phosphates was determined as shown in Fig. 1. Values are means ± SEM of three experiments. (----) The relative concentrations of inositol phosphates found in the cell suspension after 1 µM noradrenaline (see Fig. 2). * significantly different from prestimulation value ($P < 0.05$)

of inositol phosphates, it caused an increase of glucose output (Fig. 3) and lactate output (not shown) of 9.4 ± 0.5 µmol g liver^{-1} and 5.9 ± 1.7 µmol g liver^{-1}, respectively, which was very similar in extent and kinetics to that observed after 1 µM noradrenaline infusion (Fig. 3). It elicited a decrease of flow of 12.2 ± 0.6 ml g^{-1} (area under the curve, not shown), which was twice that observed after noradrenaline. All nerve-stimulation-dependent changes were qualitatively and quantitatively similar to those observed previously in this laboratory in experiments in which the non-recirculating liver perfusion was started immediately after preparation of the organ. This indicates that the liver was not damaged during the 1-h recirculating perfusion that was necessary to incorporate the *myo*-[2-^{3}H]inositol into the phosphoinositides.

DISCUSSION

It has been shown in the present study that in perfused rat liver, circulating noradrenaline (1 µM) and electrical stimulation (20 Hz, 2 ms) of the hepatic nerves, both operating via α-receptors [3–5, 11] and a cAMP-independent, probably calcium-mediated activation of glycogen phosphorylase [6, 11], enhanced glucose output to essentially the same extent and with similar kinetics but had quite different effects on inositol phosphate metabolism: Noradrenaline increased the formation of InsP_1, InsP_2 and InsP_3 clearly, while nerve stimulation enhanced the synthesis of InsP_1 slightly but had no significant influence on InsP_2 and InsP_3 (Fig. 3). The differences in inositol phosphate metabolism after neurostimulation and noradrenaline infusion could be explained by several assumptions.

a) Circulating noradrenaline might reach every hepatocyte enhancing inositol phosphate formation and thus calcium release from the endoplasmic reticulum in all parenchymal cells, while the hepatic nerves might reach only a few periportal hepatocytes directly, all other hepatocytes being stimulated indirectly by signal propagation via gap junctions. This assumption is in line with the observation that in rat liver innervation of the parenchymal cells is scarce and that conversely the density of gap junctions is high [28]. Inositol phosphate formation then would be induced in some periportal cells only and thus less inositol phosphates would be formed per gram of tissue.

b) Circulating noradrenaline and hepatic nerves might reach the same number of cells. Then, circulating noradrenaline might bind to many α-receptors on the sinusoidal aspects of the hepatocyte membrane, whereas noradrenaline released from nerve endings might bind only to the relatively few α-receptors within the nerve–hepatocyte contacts. This would lead to a more pronounced activation of inositol phosphate metabolism by circulating noradrenaline, if inositol phospholipid breakdown were proportional to the number of receptors occupied in the membrane. This was found to be the case under saturating conditions for noradrenaline, angiotensin II and vasopressin [15]: the total number of receptors increased in that order and correlated with the increasing formation InsP_3, yet the activation of glycogen phosphorylase was the same in all cases.

c) Circulating noradrenaline might cause a sustained, and nerve stimulation only a transient activation of phosphoinositide cleavage. This would elicit a higher accumulation of InsP_1, InsP_2 and InsP_3 after noradrenaline infusion. This assumption is in line with the finding that noradrenaline release from nerve endings after nerve stimulation, as measured by noradrenaline overflow into the hepatic vein [5], is indeed transient, reaching a maximum within 1–2 min and returning to basal values within 5–7 min. Another complication has to be considered on the basis of more recent findings [16, 18]. With angiotensin II and vasopressin the increase of InsP_3 was not due to a change in the Ins1,4,5P_3 levels alone, as previously assumed, but also to an alteration of the Ins1,3,4P_3 levels. Thus circulating noradrenaline and nerve stimulation might lead to the same or similar changes in the concentrations of Ins1,4,5P_3, which alone is assumed to be able to release calcium from the endoplasmic reticulum [16, 18], in spite of the major differences in the alterations of the levels of InsP_1, InsP_2 and total InsP_3.

d) Nerve endings might reach predominantly non-parenchymal cells, which then could release some signal substance that actually is responsible for the metabolic changes observed. Evidence for such a hypothesis was recently provided: the effects of nerve stimulation could be clearly reduced by inhibitors of eicosanoid synthesis [29] and could be mimicked by infusion of prostaglandins [30]. Since non-parenchymal cells represent only a minor fraction of all liver cell mass, nerve stimulation would not be expected to have a pronounced effect on the overall inositol phosphate levels in the liver homogenate.

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