SUBCELLULAR COMPARTMENTATION OF GUANINE NUCLEOTIDES AND FUNCTIONAL RELATIONSHIPS BETWEEN THE ADENINE AND GUANINE NUCLEOTIDE SYSTEMS IN ISOLATED HEPATOCYTES

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1. Introduction

The subcellular compartmentation of adenine nucleotides between the mitochondria and the cytosol has been clearly demonstrated by the use of different cell fractionation techniques [1,2]. However, data concerning the subcellular distribution of guanine nucleotides are not available.

Here, the cellular content and the subcellular distribution of guanine nucleotides has been determined in isolated rat hepatocytes using the digitonin fractionation procedure [3]. Measurements of the intracellular distribution of guanine and adenine nucleotides under selected experimental conditions reveal that under most conditions both systems are near equilibrium in the soluble but not in the mitochondrial compartment, when the mass action ratios of the nucleoside diphosphokinase and GTP-AMP phosphotransferase equilibria are considered. Some metabolic implications of these findings are discussed.

Part of this work has previously been reported in a preliminary form [4].

2. Materials and methods

Isolated hepatocytes were prepared from livers of fed Wistar rats (200–250 g) using standard procedures [5,6]. Unless otherwise mentioned, calcium-free Krebs-Henseleit bicarbonate buffer [7] was used for the final cell suspension (0.13–0.16 g wet wt/ml). The hepatocytes were disrupted and fractionated into soluble and particulate fractions using the digitonin–silicone layer technique [3], with the modifications in [8]. The fractionation medium consisted of 0.25 M sucrose, 20 mM morpholinopropane sulphonate (pH 7.2), 0.2 mM carboxyatractyloside, [U-^{14}C]sucrose (1 μCi/ml), and digitonin (2 mg/ml). Further additions were as specified in the text. Cell fractionation was started by mixing 0.5 ml cell suspension with 0.5 ml ice-cold fractionation medium. After 30 s the cells were separated by centrifugation for 15 s at 12 000 × g. Control incubations without digitonin were performed in parallel. In some experiments hepatocytes were incubated at 34°C for 30 min in a shaking water bath (40 strokes/min) before fractionation. Throughout this period the cells were continously gassed with carbogen (95% O_2, 5% CO_2). Further conditions are given in the text.

For the determination of 'adherent fluid', 0.1 ml aliquots were taken from the supernatant and corresponding pellet fractions, diluted with water to 1 ml, mixed with 10 ml Instagel (Packard Instr. Corp., Frankfurt) and measured for radioactivity in a Mark III liquid scintillation spectrometer (Searle Anal., Des Plaines, IL).

For the determination of the various nucleotides the acid pellet extracts were neutralized with 2 N KOH to pH 6.5. The nucleotides were determined enzymatically by modifying the method in [9] as follows: The reaction mixture for the determination of ATP, GTP, GDP and XTP contained (final concentrations): triethanolamine–Cl (pH 7.5) 0.15 M; magnesium acetate, 3 mM; NADP, 0.88 mM; D-glucose, 5 mM; P^1,P^5-di(adenosine-5′-)pentaphosphate, 0.05 mM; glucose-6-phosphate dehydrogenase [EC 1.1.1.49],

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0.4 U/ml. Hexokinase [EC 2.7.1.1], 0.67 U/ml; nucleoside diphosphokinase [EC 2.7.4.6], 2 U/ml; guanylate kinase [EC 2.7.4.8], 0.4 U/ml, were added sequentially (see [9]). It proved to be essential to use low amounts of hexokinase and to inhibit contaminating adenylate kinase activity by addition of P^1,P^5-di(adenosine-5'-)pentaphosphate. ADP (XDP), 5'-AMP and 5'-GMP were determined in the following assay system (final concentrations): triethanolamine–Cl (pH 7.5) 0.1 M; KCl, 48 mM; $MgSO_4$, 6 mM; NADH, 0.3 mM; phosphoenolpyruvate, 1.7 mM; lactic dehydrogenase [EC 1.1.1.27], 5.5 U/ml. Pyruvate kinase [EC 2.7.1.40], 4 U/ml; myokinase [EC 2.7.4.3], 3.6 U/ml; guanylate kinase, 0.4 U/ml, were added sequentially. An Eppendorf photometer with tenfold scale expansion or an Aminco DW-2 spectrophotometer (wavelength pair 350–375 nm) was used for enzymic–optical registration.

The cytosolic content of the various nucleotides C was calculated from the total cellular content U (digitonin absent) and the amount found in the particulate fraction P (digitonin present) according to the equation:

$$C = (T - P) / (1 - S)$$

where S represents the 'adherent fluid' (ml) carried through the silicone oil in the presence of digitonin.

The results are expressed per gram wet weight. For the wet weight determination of hepatocytes an aliquot (0.5 ml) of the final cell suspension was placed in a pre-weighed Eppendorf tube and the cells were sedimented by centrifugation (1 min at 12 000 × *g*). The supernatant was aspirated and the weight of the firmly packed cell pellet was taken as wet weight.

All biochemicals and enzymes were obtained from Boehringer, Mannheim. Carbonyl cyanide, *m*-chlorophenyl hydrazone (Cl-CCP) came from Calbiochem GmbH, Lahn. All other chemicals were of analytical grade and came from Merck, Darmstadt. [U-^{14}C]-Sucrose was obtained from Amersham Buchler, Braunschweig.

3. Results and discussion

The total guanine nucleotide content of 254 ± 8 (7) nmol/g wet wt found in freshly isolated hepatocytes was similar to that reported for total rat liver [9–11]. Like adenine nucleotides, guanine nucleotides are unevenly distributed between the soluble and particulate fraction of digitonin treated hepatocytes (table 1). However, only 22% of total guanine nucleotides, as compared to 40–45% of total adenine nucleotides were found in the particulate fraction.

Nucleoside 5'-triphosphates other than ATP and GTP were exclusively found in the soluble fraction (324 ± 28 (7) nmol/g wet wt), but could not be detected in the particulate fraction, possibly due to the limited sensitivity of the assay system used in this study. Cellular GTP amounts to ~10% of cellular nucleoside 5'-triphosphates as reported for total rat liver [10].

The subcellular concentrations of guanine nucleotides as calculated from the apparent water spaces of the two main cellular compartments (table 2), are in the range of the app. K_m values reported for enzymes using guanine nucleotides as substrates [12–16].

In experiments with freshly isolated hepatocytes from fed rats the adenine and the guanine nucleotide systems were near equilibrium, as judged from the

Table 1
Content and subcellular distribution of guanine and adenine nucleotides in isolated hepatocytes

	GTP	GDP	5'-GMP	GTP/GDP	ATP	ADP	5'-AMP	ATP/ADP
Soluble	0.155	0.043	0.014	3.94	1.25	0.31	0.11	3.97
	±0.014	±0.003	±0.002	±0.57	±0.17	±0.05	±0.02	±0.66
Particulate	0.021	0.020	0.019	1.35	0.46	0.32	0.38	1.59
	±0.004	±0.003	±0.003	±0.38	±0.06	±0.04	±0.06	±0.25

Hepatocytes (0.14–0.16 g wet wt/ml) isolated from fed rats were disrupted and fractionated as described in the experimental section. The data are expressed as μmol/g wet wt and represent means ± SEM of 7 separate experiments. For other details see section 2

Table 2
Concentrations of guanine nucleotides in subcellular fractions of isolated hepatocytes

	Cytosol (mM)	Mitochondria (mM)
GTP	0.327	0.389
GDP	0.091	0.370
5'-GMP	0.030	0.352
Σ GXP	0.448	1.111

The intracellular concentrations of the guanine nucleotides were calculated from the data of table 1 using an apparent intracellular volume of 0.476 ml/g wet wt for the cytosol, and 0.054 ml/g wet wt for the mitochondrial matrix (sucrose inaccessible 'space'). Other conditions as given in the legend to table 1

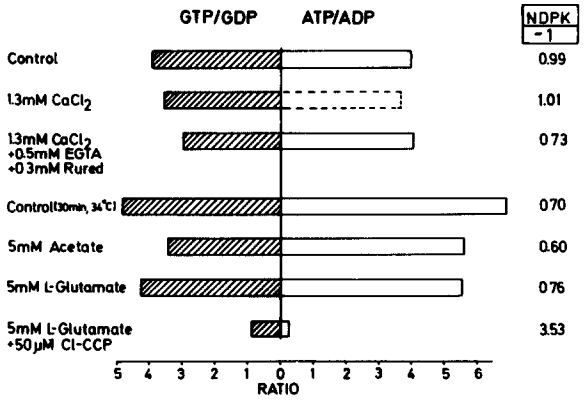

Fig.1. GTP/GDP and ATP/ADP ratios in the soluble fraction of rat hepatocytes under various experimental conditions. The corresponding data are given in tables 1, 3, except those showing the effects of calcium + and −EGTA plus ruthenium red, which are derived from experiments, where freshly isolated hepatocytes had been suspended in calcium containing buffer [7] prior to cell disruption. Where indicated, EGTA + ruthenium red (Rured) were present throughout cell fractionation at concentrations as given in the figure. Values shown are means of 3–7 separate experiments. The dash-line column indicates, that here the adenine nucleotides have been determined directly in the soluble fraction resp. supernatant after acidification and neutralisation, since the mathematical expression used for the calculation of nucleotides in this fraction is invalid under this special condition. The mass action ratio of nucleoside diphosphokinase (NDPK) has been calculated from the equation:

$$K = [GTP]\,[ADP] / [GDP]\,[ATP].$$

According to [21], the equilibrium constant for this enzyme is about 1.

mass action ratios of nucleoside diphosphokinase and GTP-AMP phosphotransferase (EC 2.7.4.10) (fig.1,2, top columns). To perturbate this equilibrium, hepatocytes were incubated in the presence of different substrates before fractionation. The results are given in table 3, fig.1 and 2. In the soluble fraction both nucleotide systems were close to equilibrium under all conditions tested, except when uncoupler (Cl-CCP) was present during incubation (fig.1). Here ATP generation by mitochondria was abolished and cytosolic GTP no longer regenerated from ATP via extramitochondrial nucleoside diphosphokinase. This is supported by the fact that no other nucleoside 5'-triphosphates could be detected under these conditions.

In the particulate fraction both nucleotide systems were considerably out of equilibrium in the presence of either $CaCl_2$, or acetate, or L-glutamate + Cl-CCP (fig.2). Exogenous Ca^{2+} (1.3 mM) when present during cell fractionation lowered the ATP/ADP ratio of the particulate fraction by ~85%, due to the hydrolysis of endogenous ATP during mitochondrial calcium uptake [17]. This effect was abolished in the presence

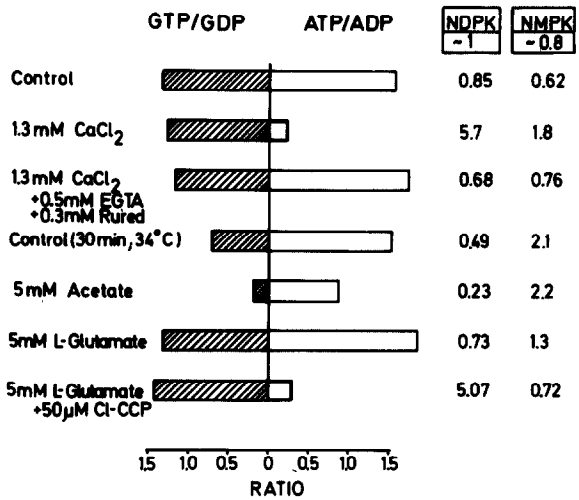

Fig.2. GTP/GDP and ATP/ADP ratios in the particulate fraction of rat hepatocytes under various experimental conditions. Experimental conditions were as given in the legend to fig.1. The mass action ratio of GTP-AMP phosphotransferase (NMPK) has been calculated from the following equation:

$$K = [ADP]\,[GDP] / [AMP]\,[GTP].$$

The equilibrium constant for this enzyme is about 0.8 [22]. More recently, a value of 1.5 has been reported [15].

Table 3
Subcellular distribution of guanine and adenine nucleotides under different experimental conditions

Condition	Fraction	GTP	GDP	5'-GMP	GTP/GDP	ATP	ADP	5'-AMP	ATP/ADP
No addition	S	0.154 ±0.007	0.032 ±0.006	0.034 ±0.012	4.87	1.267 ±0.073	0.183 ±0.061	0.139 ±0.046	6.91
	P	0.019 ±0.004	0.026 ±0.006	0.017 ±0.004	0.73	0.369 ±0.032	0.243 ±0.031	0.157 ±0.007	1.51
5 mM Acetate	S	0.129 ±0.012	0.039 ±0.006	0.028 ±0.008	3.31	1.398 ±0.214	0.249 ±0.075	0.071 ±0.028	5.61
	P	0.006 ±0.003	0.030 ±0.007	0.012 ±0.004	0.20	0.160 ±0.011	0.187 ±0.027	0.397 ±0.058	0.86
5 mM L-Glutamate	S	0.170 ±0.020	0.039 ±0.010	0.025 ±0.006	4.30	1.507 ±0.167	0.267 ±0.070	0.173 ±0.058	5.64
	P	0.031 ±0.006	0.023 ±0.011	0.017 ±0.006	1.35	0.457 ±0.046	0.246 ±0.028	0.143 ±0.007	1.86
5 mM L-Glutamate + 50 μM Cl-CCP	S	0.029 ±0.007	0.035 ±0.009	0.153 ±0.008	0.84	0.070 ±0.025	0.294 ±0.059	0.968 ±0.162	0.24
	P	0.022 ±0.008	0.015 ±0.006	0.024 ±0.005	1.47	0.104 ±0.010	0.356 ±0.036	0.339 ±0.088	0.29

Hepatocytes (0.14–0.16 g wet wt/ml) were incubated for 30 min at 34°C prior to cell fractionation under conditions as listed in the table. The data are expressed as μmol/g wet wt and represent means ± SEM of 3–5 separate experiments. S soluble, P particulate. Other experimental details were as given in section 2

of EGTA + ruthenium red, compounds preventing mitochondrial calcium sequestration [18]. The GTP/GDP ratio however, was not affected under the conditions (fig.2). In the presence of L-glutamate + Cl-CCP, high GTP levels were maintained in the particulate fraction due to an enhanced substrate chain phosphorylation under these conditions and a limited capacity of intramitochondrial nucleoside diphosphokinase. However, in the presence of acetate a significant decrease of the particulate GTP/GDP ratio was observed (fig.2). During acetate activation AMP is generated in the mitochondrial matrix but cannot leave this compartment. AMP has to be rephosphorylated to ADP by the GTP-AMP phosphotransferase [19] resulting in the observed drop of the GTP/GDP ratio. Apparently, under these conditions GTP formation via succinic thiokinase (EC 6.2.1.4) and mitochondrial nucleoside diphosphokinase [20] is not sufficient to maintain the GTP concentration seen in the absence of acetate.

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