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CYCLIC AMP-DEPENDENT PHOSPHORYLATION IN THE CONTROL OF BIOTRANSFORMATION IN THE LIVER

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Abstract—The possibility of a short-term cAMP-dependent regulation of mixed-function oxidation and of glucuronide formation was investigated in isolated mouse hepatocytes and in mouse liver microsomal membranes. N^6, O^2-dibutyryl cAMP (in accordance with its increasing effect on gluconeogenesis) decreased aminopyrine oxidation and *p*-nitrophenol conjugation in isolated hepatocytes, while the phenolphthalein conjugation remained unaltered. Similar to dibutyryl cAMP the Ca^{2+} ionophore A 23187 also decreased aminopyrine oxidation. In cell-free systems the phosphorylation of isolated microsomal membranes by the exogenous cAMP-dependent protein kinase was inhibitory on aminopyrine oxidation and *p*-nitrophenol glucuronide formation but aniline oxidation and phenolphthalein glucuronidation were not affected. The correlation between the negative cAMP-dependent control of certain processes of biotransformation and the positive cAMP-dependent regulation of gluconeogenesis is discussed.

The processes of biotransformation in the liver are responsible for detoxification and elimination of a wide variety of drugs and several endogenous compounds. Biotransformation also represents a metabolic load for the hepatic intermediary metabolism (for review see [1–3]), thus, to understand its regulation is of special interest. The well-characterized induction of enzyme components catalyzing the reactions of biotransformation by various inducers is a slow form of regulation known to be affected by various dietary factors [4]. However, increased drug oxidation has been shown to cause prompt metabolic changes, e.g. an immediate large increase in hepatic oxygen consumption [5], NADPH demand in hepatocytes, decreased glucose production etc., which are also dependent on the diet or starvation [1, 2, 6]. In spite of the observation by Fouts from 1962 [7] on the depressing effect of catecholamines on biotransformation little is known about the short-term hormonal regulation of drug metabolism. Our knowledge on the short-term regulatory types was restricted mainly to competing reactions for substrates and cofactors (e.g. NADPH) required both for biotransformation of various drugs and for major pathways of intermediary metabolism (gluconeogenesis, fatty acid synthesis etc.) [1].

During starvation hepatic gluconeogenesis is the main source of blood glucose. It is well-known that gluconeogenesis is under positive cAMP-dependent regulation in the liver, and the role of protein phosphorylation in the hormonal control of metabolic pathways is evidenced (for recent review see [8]). Several reports have been published recently on the phosphorylation of certain forms of purified hepatic microsomal cytochrome P-450 (main components of the mixed-function oxidase system responsible for drug oxidation) by the catalytic subunit of cAMP-dependent protein kinase accompanied by a decrease in the reconstituted monooxygenase activity [9–11]. Moreover, the addition of N^6, O^2-dibutyryl cyclic AMP has been shown to decrease the content of hepatic cytochrome P-450 [12, 13]. Based on these results and on the close correlation observed between the magnitude of decrease in gluconeogenesis and the increased rate of drug oxidation [6] it was tempting to suppose that oxidation of certain drugs is under control of cAMP-dependent phosphorylation in hepatocytes and this is in a close interrelationship with the regulation of gluconeogenesis. However, in most cases drug oxidation is only the first phase of biotransformation followed by a second conjugation phase, which is frequently glucuronide formation catalysed by different UDP-glucuronosyl transferases (for review see [14]). It requires UDP-glucuronic acid and also represents a significant metabolic load for the hepatic intermediary metabolism.

In this work a cAMP-dependent short-term regulation was investigated in two major routes of drug metabolizing processes: in mixed-function drug oxidation, which is a dominant pathway in the preparatory phase I of biotransformation [1] and also in glucuronidation, which is quantitatively the most important conjugation phase II reaction [14]. It was shown that dibutyryl cAMP decreased aminopyrine oxidation in isolated hepatocytes in a close correlation with the increase of gluconeogenesis and it also inhibited *p*-nitrophenol conjugation. The combined addition of catalytic subunit of cAMP-dependent protein kinase and ATP inhibited aminopyrine oxidation and *p*-nitrophenol glucuronidation in microsomal membranes indicating that the negative control of cAMP on biotransformation is exerted via the well-known mechanisms of cAMP-dependent

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protein phosphorylation. On the other hand, oxidation of aniline and conjugation of phenolphthalein were not decreased. Gluconeogenesis is also stimulated by a Ca^{2+}-dependent regulatory process. In order to demonstrate a correlation between the positive control of gluconeogenesis and the negative control of biotransformation the effects of Ca^{2+} ionophore A 23187 were also investigated in isolated hepatocytes.

MATERIALS AND METHODS

Chemicals. Collagenase, type IV and uridine 5′-diphosphoglucuronic acid sodium salt were purchased from Sigma Chemical Co. A 23187 and dibutyryl cyclic AMP from Calbiochem. All chemicals used were of analytical grade.

Isolated hepatocytes from CFLP male mice (25–30 g body weight) fed *ad libitum* or starved 24 hr prior to the experiments as indicated were prepared with the collagenase perfusion method as detailed earlier [15]. The hepatocytes (5×10^6 cells/ml) were incubated in Krebs–Henseleit bicarbonate buffer pH 7.4 containing 1% albumin, 8.5 mM glucose, 5 mM pyruvate and amino acids necessary for protein synthesis (1 mM of each) under constant bubbling of gas (O_2:CO_2, 95:5 v/v) at 37°. In cases when the cells were prepared from fasted animals glucose, pyruvate and amino acids were omitted from the incubation medium and lactate in concentrations as indicated was used as gluconeogenic substrate.

Aminopyrine oxidation in hepatocytes was measured with the detection of 4-aminoantipyrine, the *N*-demethylated product of aminopyrine [16] as described elsewhere [6].

Glucose production of the cells was measured by the glucose oxidase method [17].

Conjugation of *p*-nitrophenol and phenolphthalein in isolated hepatocytes was determined on the basis of disappearance of aglycones. Incubations were stopped by the addition of ice-cold ethanol. The samples were centrifuged and the decrease of *p*-nitrophenol and phenolphthalein content were monitored in the supernatants [18, 19].

Microsomal membranes were prepared from isolated hepatocytes as described by Pilkis *et al.* [20]. The dissociated catalytic subunit of cAMP-dependent protein kinase was prepared from rabbit skeletal muscle. The purification procedure was essentially the same as detailed previously [21]. Briefly, rabbit skeletal muscle was homogenized in a medium containing 0.04 M KCl, 0.02 M $MgCl_2$, 0.01 M Tris–HCl buffer (pH 7.5) and the protease inhibitor phenylmethylsulphonyl fluoride (2 mM). The crude extract was prepared and a DEAE-cellulose chromatography was carried out in the presence of cAMP as described previously [22]. Fractions containing the dissociated catalytic subunit were pooled, dialyzed against 5 mM Tris–HCl buffer (pH 7.5) and applied onto a phosphocellulose column (0.7×3.0 cm). Elution was performed by an NaCl concentration gradient in 10 mM Tris–HCl buffer (pH 7.5). The catalytic subunit was eluted at about 0.2 M NaCl. The specific activity of the preparation was 50–120 nmole phosphate transferred $\times$ min^{-1} $\times$ mg $protein^{-1}$ when it was measured with H2b histone as substrate. The heat stable inhibitor protein was prepared according to the method of Ashley and Walsh [23]. The amount of inhibitor used in the incubation mixture for membrane phosphorylation decreased the activity of the catalytic subunit by more than 95% when it was measured with H2b histone as substrate.

Microsomal membranes (1 mg protein/ml) were incubated and aminopyrine oxidation was measured essentially as reported by Lieber *et al.* [24] by the determination of formaldehyde by Nash [25]. Aniline oxidation was examined by the determination of *p*-aminophenol by Schenkman *et al.* [26].

For the determination of UDP-glucuronosyl transferase activities toward *p*-nitrophenol and phenolphthalein microsomal membranes were incubated as described by Bock *et al.* [27]. Ten to twenty μg protein of catalytic subunit preparation/ml incubation mixture was applied when indicated.

DNA content of isolated hepatocytes were measured according to Burton [28]. Protein content was determined by Lowry *et al.* [29].

RESULTS

Effect of dibutyryl-cAMP and Ca^{2+} *ionophore* A 23187 *on aminopyrine oxidation and on gluconeogenesis in isolated hepatocytes*

Interrelationship between aminopyrine oxidation and gluconeogenesis was investigated in isolated hepatocytes prepared from 24-hr starved mice with simultaneous measurement of glucose production and aminopyrine *N*-demethylation. Aminopyrine oxidation and gluconeogenesis were linear in time in the course of a 30 min incubation (data not shown). In case of lactate, used as gluconeogenic substrate, maximal glucose production could be obtained with the addition of 10 mM lactate [6]. It was noted that in the presence of 10 mM lactate and 1 mM aminopyrine the rate of aminopyrine oxidation was also maximal. In agreement with our, and other, earlier results [6, 2] aminopyrine inhibited glucose production from lactate both at suboptimal (1 mM lactate) or at optimal substrate supply (10 mM) (Table 1). In the presence of dibutyryl cAMP or A 23187 the balance between gluconeogenesis and aminopyrine oxidation was changed. Both 10^{-4} M dibutyryl cAMP and 10^{-5} M A 23187 enhanced gluconeogenesis from 1 mM lactate by 37% and 68%, respectively. At the same time both dibutyryl cAMP and A 23187 decreased the rate of aminopyrine *N*-demethylation by about 20% and 44%, respectively. Decreased glucose production caused by 1 mM aminopyrine was enhanced by 60% in the presence of 10^{-4} M dibutyryl cAMP and by about 190% in the presence of 10^{-5} M A 23187. Similar results could be obtained when the same experiments were performed using 10 mM lactate as gluconeogenic precursor. These results also showed that the inhibitions of gluconeogenesis caused by aminopyrine were moderated by dibutyryl cAMP and A 23187. The inhibitory effect of A 23187 on aminopyrine *N*-demethylation was dependent on the presence of Ca^{2+}; in calcium-depleted hepatocytes no inhibitory effect of A 23187 could be detected, while dibutyryl cAMP inhibited aminopyrine oxidation as in the

Table 1. Effect of dibutyryl-cAMP and A 23187 on gluconeogenesis and aminopyrine oxidation in isolated hepatocytes prepared from starved mice

Addition	Lactate	Glucose production (nmole/g liver/min) Control			1 mM aminopyrine			4-aminoantipyrine formation (nmole/g liver/min)		
None	1 mM	150 ± 14	(17)	100%	30 ± 5	(8)	100%	57.6 ± 3.6	(14)	100%
10^{-4} M dibutyryl-cAMP	1 mM	206 ± 19	(8)	137%	48 ± 9	(8)	160%	44.8 ± 6.3	(7)	79.7%
10^{-5} M A 23187	1 mM	253 ± 36	(6)	169%	88 ± 6	(6)	293%	31.7 ± 3.5	(6)	56.4%
None	10 mM	228 ± 20	(8)	100%	64 ± 32	(8)	100%	85.6 ± 5.1	(8)	100%
10^{-4} M dibutyryl-cAMP	10 mM	311 ± 49	(7)	136%	125 ± 50	(7)	195%	76.9 ± 6.3	(7)	89.8%
10^{-5} M A 23187	10 mM	296 ± 30	(4)	130%	174 ± 30	(4)	271%	58.9 ± 10.1	(8)	68.8%

Isolated hepatocytes prepared from 24 hr starved mice were incubated in incubation buffer supplemented with lactate for 30 min. The incubations were performed in the presence or in the absence of 1 mM aminopyrine and the effect of dibutyryl-cAMP and A 23187 was investigated on glucose production and 4-aminoantipyrine formation. Means ± SD. (N).

presence of Ca^{2+} (data not shown). Combined addition of 10^{-5} M A 23187 and 10^{-4} M dibutyryl cAMP did not cause a more pronounced inhibition (67.8 ± 5.7% (N = 4) in the presence of 1 mM aminopyrine using 10 mM lactate as gluconeogenic precursor) beyond the inhibitory effect of 10^{-5} M A23187 alone (Table 1). The inhibition of aminopyrine *N*-demethylation by dibutyryl cAMP (Fig. 1) and by A 23187 (data not shown) was also observed in isolated hepatocytes prepared from fed mice.

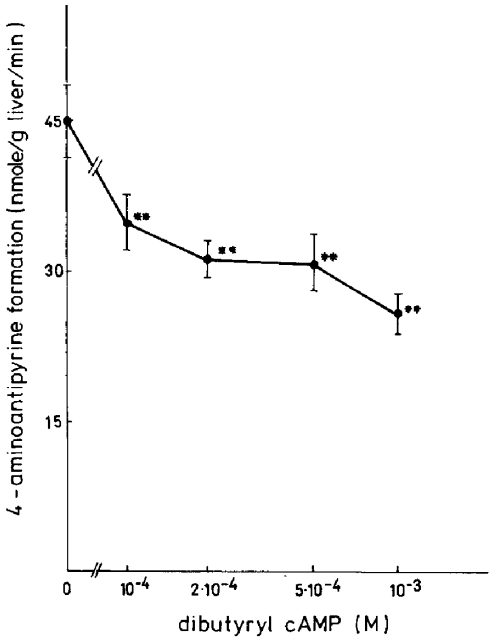

Fig. 1. Inhibitory effect of dibutyryl cAMP on aminopyrine oxidation in isolated hepatocytes prepared from fed mice. Isolated hepatocytes prepared from fed mice were incubated in the presence of dibutyryl cAMP for 30 min. The formation of 4-aminoantipyrine was measured. The concentration of aminopyrine was 1 mM. Mean ± SD (N = 4). *Different from control at $P < 0.01$ level.

Effect of dibutyryl cAMP on p-*nitrophenol and phenolphthalein conjugation of isolated hepatocytes*

Isolated hepatocytes were incubated in the presence of *p*-nitrophenol or phenolphthalein. Maximal rates of conjugation were reached at 100 μM substrate concentrations assuring a constant rate of conjugation throughout a 120 min incubation (Table 2). At these substrate concentrations mainly glucuronide conjugates, and only at a lesser extent sulfate conjugates, are formed according to the observations gained in isolated rat hepatocytes [30]. D-Galactosamine has been shown to inhibit a series of uridylate-dependent processes [31, 32], among others UDP–glucuronic acid formation [33] and glucuronidation [34, 35]. 10 mM D-galactosamine decreased the *p*-nitrophenol- and phenolphthalein-conjugation in isolated hepatocytes (Table 2) indicating that glucuronide conjugates are formed.

The addition of dibutyryl cAMP caused a concentration-dependent decrease of *p*-nitrophenol conjugation (Fig. 2A). At the same time phenolphthalein conjugation was not affected even in the presence of 10^{-3} M db cAMP (Fig. 2B).

The inhibitory effects of dibutyryl cAMP on aminopyrine oxidation and *p*-nitrophenol conjugation raised the possibilities that (i) dibutyryl cAMP known to change the balance between several pathways of the intermediary metabolism (substrate or cofactor supply, etc.) influenced indirectly both drug oxidation and conjugation; (ii) cAMP-dependent control was directly involved in regulation of enzyme activities participating in aminopyrine oxidation or *p*-nitrophenol glucuronidation. To tackle this problem experiments in cell-free systems were undertaken.

Effect of the dissociated catalytic subunit of cAMP-dependent protein kinase on aminopyrine N-*demethylase, aniline hydroxylase and on* p-*nitrophenol- and phenolphthalein-UDP–glucuronosyl transferase activities in microsomal membranes*

Microsomal membranes were prepared from isolated hepatocytes and the rate of aminopyrine and aniline oxidation was investigated in the presence of the exogenous catalytic subunit of cAMP-dependent

Table 2. Effect of D-galactosamine on *p*-nitrophenol and phenolphthalein conjugation

Addition	*p*-Nitrophenol disappearance (nmole/g liver/min)	Phenolphthalein disappearance (nmole/g liver/min)
None	44.7 ± 3.9 (4)	30.8 ± 10.5 (6)
10^{-2} M D-galactosamine	24.0 ± 2.1* (4)	4.2 ± 2.1* (4)

Isolated hepatocytes were prepared from fed mice and incubated in incubation buffer supplemented with 100 μM *p*-nitrophenol or 100 μM phenolphthalein for 30 min. The disappearance of *p*-nitrophenol and phenolphthalein were measured. Mean ± SD.
* Different from control at $P < 0.01$ level.

protein kinase, ATP and the heat-stable protein inhibitor of cAMP-dependent protein kinase. The addition of ATP alone resulted in approximately 25% inhibition of aminopyrine oxidation in microsomal membranes in accordance with other observations [36]. Approximately 50% inhibition occurred when the catalytic subunit of cAMP-dependent protein kinase was also present throughout the incubation (Table 3). The combined addition of heat-stable protein inhibitor of cAMP-dependent protein kinase with catalytic subunit and ATP prevented the inhibitory effect by the catalytic subunit. The heat-stable inhibitor protein also counteracted the inhibition of aminopyrine oxidation by ATP alone. cAMP was found to be ineffective on aminopyrine oxidation (data not shown). No inhibition of aniline hydroxylation was observed by the catalytic subunit and ATP, or ATP alone (data not shown).

Glucuronidation of *p*-nitrophenol and phenolphthalein was also investigated in microsomal membranes prepared from isolated hepatocytes in the presence of exogenous UDP–glucuronic acid. *p*-Nitrophenol–UDP–glucuronosyl transferase activity measured in the microsomal fraction in the presence of 0.5 mM *p*-nitrophenol was linear in time in the course of a 30 min incubation, and no alteration was found in the presence of cAMP (data not shown). The addition of 5 mM ATP caused about a 25% inhibition of *p*-nitrophenol glucuronidation (Table 3) in agreement with previous data gained in other systems [37], while the combined addition of ATP with the catalytic subunit of cAMP-dependent protein kinase induced about a 50% decrease. The addition of the heat-stable inhibitor protein of the cAMP-dependent protein kinase completely reverted the inhibitory effect of the catalytic subunit. The rate of phenolphthalein glucuronidation was not altered in the presence of ATP or ATP plus catalytic subunit (data not shown).

DISCUSSION

Several cytochrome P-450 isoenzymes participate in mixed-function oxidation in phase I, and also

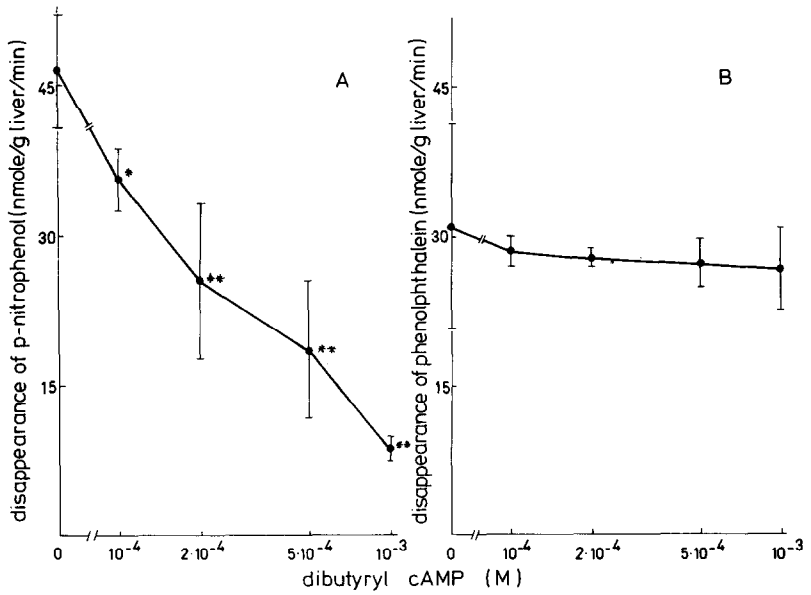

Fig. 2. Effect of dibutyryl cAMP on *p*-nitrophenol and phenolphthalein, conjugation in isolated hepatocytes. Isolated hepatocytes prepared from mice fed *ad libitum* were incubated for 30 min in the presence of 100 μM *p*-nitrophenol (A) or of 100 μM phenolphthalein (B). The effect of dibutyryl cAMP was investigated on conjugation. Mean ± SD. N = 4. *Different from control at $P < 0.05$; **at $P < 0.01$ level.

Table 3. Effect of free catalytic subunit of cAMP-dependent protein kinase on *p*-nitrophenol conjugation and on aminopyrine oxidation in microsomal membranes prepared from isolated hepatocytes

Addition	*p*-Nitrophenol glucuronide (nmole/mg protein/min)	Formaldehyde formation
None	22.82 ± 2.08 (9)	1.76 ± 0.39 (6)
$5 \cdot 10^{-3}$ M ATP	17.03 ± 2.03† (6)	1.36 ± 0.20* (6)
C	20.27 ± 1.58 (4)	1.55 ± 0.28 (4)
I	21.06 ± 2.02 (4)	1.82 ± 0.16 (4)
$5 \cdot 10^{-3}$ M ATP + C	13.68 ± 1.39§ (6)	0.91 ± 0.10§ (6)
$5 \cdot^{-3}$ M ATP + I	19.63 ± 1.05§ (4)	1.68 ± 0.12‡ (4)
$5 \cdot 10^{-3}$ M ATP + C + I	17.80 ± 0.71¶ (4)	1.27 ± 0.36‖ (4)

Isolated hepatocyte microsomal membranes were incubated for 10 min in the presence of 0.5 mM *p*-nitrophenol and 3 mM UDP-glucuronic acid or 5 mM aminopyrine. Free catalytic subunit of cAMP-dependent protein kinase (C), or its heat-stable protein inhibitor (I) was added as indicated and *p*-nitrophenol glucuronide formation or formaldehyde formation was determined. Mean ± SD (N).

* Different from the control at $P < 0.05$; †at $P < 0.01$ level.

‡ Different from ATP treated at $P < 0.05$; § at $P < 0.01$ level.

‖ Different from ATP + C treated at $P < 0.05$; ¶ at $P < 0.01$ level.

different UDP-glururonosyl transferases catalyse the glucuronidation in phase II of biotransformation. In phase I the oxidation of aminopyrine, in phase II the glucuronidation of *p*-nitrophenol, were shown to be under negative cAMP-dependent control. The inhibitory effect of the catalytic subunit of cAMP-dependent protein kinase on the aminopyrine *N*-demethylase activity and also on *p*-nitrophenol UDP–glucuronosyl transferase activity in cell free systems (Table 3) in agreement with the inhibition of aminopyrine oxidation and *p*-nitrophenol conjugation by dibutyryl cAMP in isolated hepatocytes (Figs 1 and 2; Table 1) demonstrate that this negative control is mediated by the cAMP-dependent protein kinase. The observations on this negative regulation of mixed-function oxidation in phase I are in agreement with results obtained with various forms of purified soluble cytochrome P-450s; the reconstituted monooxygenase activities were decreased after phosphorylation by cAMP-dependent protein kinase [9–11], while to our knowledge similar results concerning the UDP–glucuronosyl transferase activities have not been described. These results suggest that a short term cAMP-dependent regulation exists in both phases of biotransformation.

Besides aminopyrine oxidation and *p*-nitrophenol glucuronidation the oxidation of aniline and the glucuronidation of phenolphthalein were measured. A unique liver cytochrome P-450 isoenzyme, 3a (also called P-450j)—different from the extensively-studied major hepatic cytochrome P-450 families—exhibits high catalytic activity in aniline hydroxylation [38–40]. There are several reports on the measurable catalytic activities of cytochrome P-450 3a but aminopyrine does not belong to its substrates [38, 41]. The substrate specificities of various UDP–glucuronosyl transferases are also different and frequently overlapping; *p*-nitrophenol- and phenolphthalein glucuronosyl transferase are different enzymes [27, 42, 43]. Contrary to aminopyrine *N*-demethylase aniline hydroxylase activity was not decreased by ATP and the catalytic subunit of cAMP-dependent protein kinase in microsomal membranes. In agreement with these data dibutyryl cAMP did not decrease aniline oxidation in isolated hepatocytes (G. Bánhegyi *et al.*, unpublished observations). While *p*-nitrophenol glucuronidation is decreased, phenolphthalein UDP–glucuronosyl transferase activity is not altered by the catalytic subunit of cAMP-dependent protein kinase in microsomal membranes and in accordance with these observations gained in cell-free systems dibutyryl cAMP does not decrease phenolphthalein conjugation in isolated hepatocytes (Fig. 2B); it is inhibitory only on *p*-nitrophenol conjugation (Fig. 2A). Thus, the cAMP-dependent negative regulation in both the mixed-function oxidation and glucuronidation seems to be selective; there are cytochrome P-450 isoenzymes and UDP–glucuronosyl transferases, which are not under cAMP-dependent control.

Our results show a close correlation between the cAMP-dependent positive control of gluconeogenesis and the cAMP-dependent negative control of drug oxidation in isolated hepatocytes (Table 1). (This trend is even more emphasized in experiments with Ca^{2+} ionophore A 23187. Thus the positive control of gluconeogenesis is manifested in a Ca^{2+}-dependent way, too.) cAMP is known to be an ancient hunger signal. The priority of glucose production in the liver is proven through several cAMP-dependent regulatory mechanisms. Positive regulation of gluconeogenesis may be further supported by cAMP-dependent negative regulatory mechanisms as well. These negative regulations on one hand may preserve substrates, e.g. amino acids for glucose production among others through the cAMP-dependent decrease of protein synthesis [44, 45]; or on the other hand may save gluconeogenic intermediates (glucose-6-phosphate etc.) from diverting away to other pathways, namely to NADPH supply for mixed-function oxidation of drugs [1] or to UDP–glucuronic acid synthesis for conjugation reactions. It is of special interest that cytochrome P-450 3a

(not under negative cAMP-dependent control) is not depressed but even induced by fasting [46] and its importance in starvation has been proposed [47].

The Ca^{2+} mediator system is also known to act in the direction of increased glucose production. Our finding—the Ca^{2+} ionophore A 23187 inhibits 4-amino antipyrine formation in isolated hepatocytes (Fig. 1)—supports the presumption: priority of glucose formation under certain conditions may be ensured also by the negative control of some gluconeogenic intermediates consuming processes.

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