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Production, Uptake, and Metabolic Effects of Cyclic AMP in the Bivascularly Perfused Rat Liver

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ABSTRACT. Production, uptake, and metabolic effects of cyclic AMP (cAMP) were measured in the bivascularly perfused rat liver in anterograde and retrograde perfusion. Glucagon, cAMP, N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP were infused into the portal vein (anterograde perfusion), the hepatic vein (retrograde perfusion), or the hepatic artery (anterograde and retrograde perfusion) in order to reach different cell populations. The following results were obtained: (1) cAMP release caused by glucagon was directly proportional to the cell spaces that were accessible via the hepatic artery in anterograde and retrograde perfusion; since the metabolic effects of glucagon were not proportional to the accessible cell spaces, this observation also implies a disproportion between cAMP release and metabolic effects of the hormone; (2) when cAMP and N^6,2′-*O*-dibutyryl cAMP were given to all liver cells (e.g. when infused into the portal vein), their metabolic effects were qualitatively and quantitatively the same and qualitatively equal to the effects of glucagon; (3) the changes caused by cAMP were a function of the cell spaces that can be reached via the hepatic artery in anterograde and retrograde perfusion; this behaviour contrasts markedly with that of glucagon, whose metabolic effects were practically independent of the accessible cell spaces; and (4) the effects of N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP were independent of the cell spaces that were accessible via the hepatic artery in anterograde and retrograde perfusion; in this respect their behaviour was equal to that of glucagon. It is apparent that exogenously added cAMP mimicked the metabolic effects of glucagon in the liver only when it was supplied to all liver cells. Since glucagon, N^6,2′-*O*-dibutyryl cAMP, and N^6-monobutyryl cAMP were able to produce a full response even when given to only 30% of the liver parenchyma, it was concluded that cAMP production under the stimulus of glucagon or in consequence of the metabolic transformation of N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP occurs in a compartment to which exogenous cAMP has no access. cAMP generated within this compartment is possibly able to diffuse from cell to cell. BIOCHEM PHARMACOL **54**;10: 1115–1125, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. bivascular liver perfusion; cAMP; N^6,2′-*O*-dibutyryl-cAMP; N^6-monobutyryl-cAMP; glycogenolysis; glycolysis; oxygen uptake

Work performed in our laboratory revealed that when glucagon was infused into the hepatic artery in anterograde and retrograde bivascular perfusion of the rat liver, the effects of the hormone on glycogenolysis, oxygen uptake, and glycolysis were not a function of the accessible cellular spaces [1]. Irrespective of the perfusion mode and infusion route (portal and hepatic vein or hepatic artery), the metabolic effects were practically the same, in spite of the fact that maximally 30% of the total cellular space is accessible through the hepatic artery in retrograde perfusion (hepatic vein → portal vein) [1, 2]. Thus, the action of glucagon exceeds the accessible cell space when this hormone is introduced into the hepatic artery in retrograde perfusion. This could be due to a zonation of the action of glucagon, but there are several arguments that seem to exclude this possibility [1, 3]. For this reason, the phenomenon has been interpreted as reflecting some kind of cell-to-cell transmission of the glucagon signal.

As an extension of our previous work, we are now presenting the results of a series of experiments in which the action of cAMP† was investigated in the bivascularly perfused liver. Basically, we have measured the stimulation of cAMP production by glucagon when this hormone was infused into the hepatic artery and the effects of cAMP plus two structural analogues infused into the same vessel in anterograde and retrograde perfusion. cAMP release by the perfused liver has long been used as an indicator for adenylate cyclase stimulation caused by glucagon [4]. Furthermore, in the once-through perfused liver, cAMP is a full agonist when infused into the portal vein in anterograde perfusion, a fact known since 1971 [5] and confirmed

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† *Abbreviation:* cAMP, cyclic AMP.

thereafter by several workers [6–9]. Consequently, it should be equally possible to investigate the effects of cAMP on the different cell populations that can be reached when substances are infused into the hepatic artery by means of anterograde and retrograde perfusion [1].

The present study was performed with two main questions in mind. The first one concerns the glucagon-stimulated cAMP release: is it a function of the accessible cell spaces or is it proportional to glycogenolysis stimulation? The second question is one about the metabolic effects of exogenously added cAMP and its analogues, N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP. In principle, the effects of these compounds on the different cell populations that can be reached via the hepatic artery in anterograde and retrograde perfusion should be equal to those of glucagon. Analysis of eventual differences in behaviour, however, may contribute to further elucidating the mechanism of action of glucagon in the liver.

MATERIALS AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. All enzymes and coenzymes used in the enzymatic and radiochemical assays, cAMP, N^6,2′-*O*-dibutyryl cAMP, and N^6-monobutyryl cAMP were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]Water was purchased from E. I. Du Pont de Nemours (Boston, MA, U.S.A.). [8-^{3}H]cAMP (29 Ci/mmol) was purchased from Amersham International (Buckinghamshire, U.K.).

Liver Perfusion

Male albino rats (Wistar), weighing 180–220 g, were fed *ad lib.* with a standard laboratory diet (Purina®). For the surgical procedure, the rats were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg).

Hemoglobin-free, non-recirculating bivascular liver perfusion was performed either in the anterograde mode (entry via the portal vein plus hepatic artery and exit via the hepatic vein) or in the retrograde mode (entry via the hepatic vein plus hepatic artery and exit via the portal vein). The surgical technique has been described elsewhere [2]. *In situ* perfusion was performed, the flow being maintained constant by two peristaltic pumps. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37°). The flow through the portal vein (anterograde) or the hepatic vein (retrograde) was between 28 and 32 mL/min. The flow through the hepatic artery was between 2 and 3 mL/min. All perfusion experiments were initiated in the anterograde mode. Retrograde perfusion was established by changing the direction of flow at 5 min before sampling of the effluent perfusate was initiated.

Analysis of Data

Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for glucose, lactate, and pyruvate by standard enzymatic procedures [10–12]. The oxygen concentration in the outflowing perfusate was monitored continuously, employing a teflon-shielded platinum electrode positioned in a plexiglass chamber at the exit of the perfusate.

cAMP in the outflowing perfusate was measured by means of isotopic competition between [8-^{3}H]adenosine 3′,5′cyclic monophosphate and cAMP for the protein kinase binding site, essentially according to the procedure described by Brown *et al.* [13].

Metabolic rates were calculated from input–output differences and the total flow rates and were referred to the wet weight of the liver or to the intracellular space accessible in each perfusion mode [1].

Treatment of Data

The statistical significance of the differences between parameters was evaluated by means of the Primer program, version 1.0 (MacGraw–Hill). Student's *t*-test, the paired Student's *t*-test, and the Student–Newman–Keuls test were applied according to the context, and the results are given in the text as the *P* values. $P < 0.05$ was adopted as a criterion of significance. The times required for half-maximal effects ($t_{max/2}$) of cAMP, glucagon, N^6,2′-*O*-dibutyryl cAMP, and N^6-monobutyryl cAMP on glucose release from endogenous glycogen were calculated by means of Lagrange's interpolation formula, which, when adapted to the particularities of the present work, becomes [14]:

$$t_{max/2} = \sum_{i=1}^{n} t_i \prod_{\substack{j=1 \\ j\neq i}}^{n} (x_{max}/2 - x_j) \Bigg/ \prod_{\substack{j=1 \\ j\neq i}}^{n} (x_i - x_j) \qquad (1)$$

In equation (1), t_i represents the different collection times after the onset of the agonist infusion, x_i or x_j the various rates of glucose release, and x_{max} the final new steady-state level of glucose release under the influence of the agonist.

RESULTS

Glucagon-Stimulated cAMP Release

The release of cAMP in the experiments performed was measured according to four different experimental protocols: (A) anterograde perfusion and glucagon in the hepatic artery; (B) retrograde perfusion and glucagon in the hepatic artery; (C) anterograde perfusion and glucagon in the portal vein; and (D) retrograde perfusion and glucagon in the hepatic vein. The rate of glucagon infusion was the same in all experiments, namely 35 pmol min^{-1} g^{-1}. This infusion rate produces a sinusoidal concentration of approximately 10 nM. This saturating concentration was chosen in order to minimize the effects of concentration gradients along the sinusoidal bed. Figure 1 shows the time–courses of cAMP

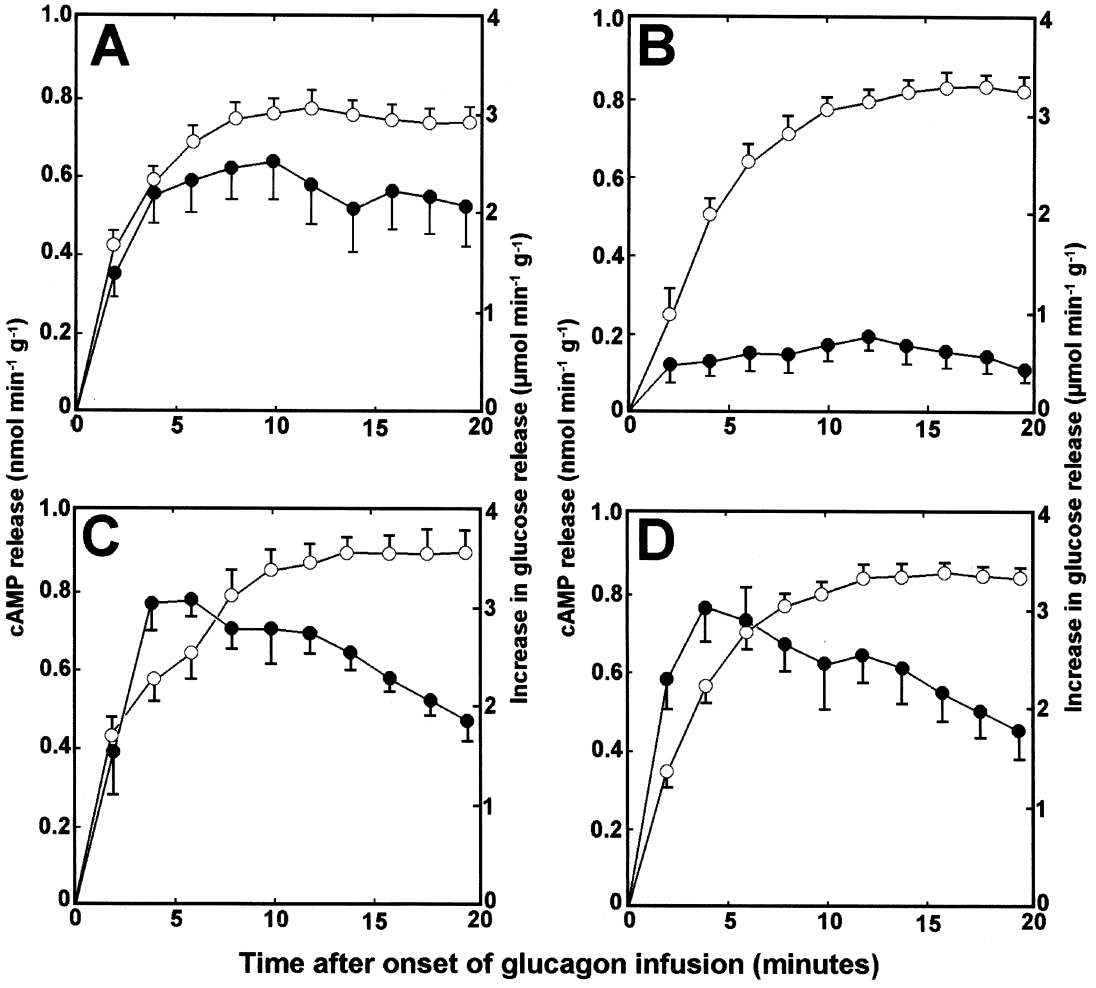

FIG. 1. Time–courses of cAMP (●——●) and glucose (○——○) release in the bivascularly perfused rat liver during glucagon infusion. Livers from fed rats were perfused bivascularly either in the anterograde or retrograde modes as described in Materials and Methods. Glucagon was infused at a mean rate of 35 pmol min^{-1} g^{-1} during 20 min. cAMP appearing in the effluent perfusate was determined by means of a radiochemical assay. Glucose was measured enzymatically. The data points are means ± SEM of 4–9 liver perfusion experiments. (A) Anterograde perfusion, glucagon in the hepatic artery; (B) retrograde perfusion, glucagon in the hepatic artery; (C) anterograde perfusion; glucagon in the portal vein; and (D) retrograde perfusion, glucagon in the hepatic vein.

release in each of the four experimental protocols. Glucose release (glycogenolysis) is also shown in order to allow a comparison between cAMP release and the metabolic effects of glucagon.

Figure 1 revealed that the rates and the kinetics of cAMP release are dependent on both the perfusion mode and the infusion route of glucagon. This is in sharp contrast with glucose release, which increases similarly for all conditions, the same being true for oxygen consumption stimulation and glycolysis inhibition, as shown elsewhere [1]. Similar rates and kinetics of cAMP release were found when glucagon entered the portal vein and the hepatic vein, as shown by panels C and D of Fig. 1. In both cases, there was a maximum between 4 and 6 min after the onset of glucagon infusion, followed by a steady decline during the rest of the infusion time. At 20 min, the rate of cAMP release was approximately half that of the peak value. When glucagon was infused into the hepatic artery, either via anterograde (Fig. 1A) or retrograde (Fig. 1B) perfusion, the rates of cAMP release did not show a well-defined maximum. Thus, the time–course of release was substantially different when compared with the time–course of release that was observed when glucagon was infused into the portal or hepatic vein (Fig. 1, C and D). Additionally, in the retrograde mode, much less cAMP was released.

In Fig. 2, the rates of cAMP release were expressed as nmol min^{-1} (mL accessible cell space)$^{-1}$. This was accomplished by dividing the rates expressed as nmol min^{-1} g^{-1} by the corresponding accessible cell spaces (expressed as mL/g). The latter were determined in a previous work by means of the multiple-indicator dilution technique, using [^{3}H]sucrose and [^{3}H]water as indicators for the extracellular and total spaces, respectively [1]. The differences in mean transit times of both indicators were used for calculating the

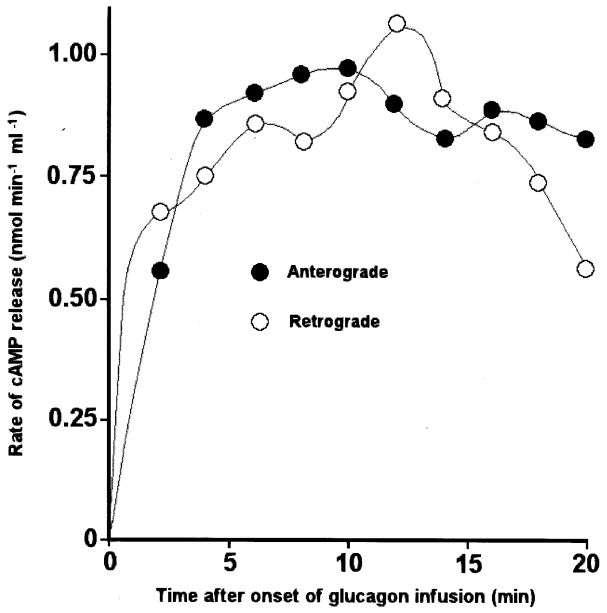

FIG. 2. Rates of cAMP release due to glucagon infusion into the hepatic artery in anterograde and retrograde perfusion, corrected for the accessible cellular spaces. Data already shown in panels A and B of Fig. 1 were recalculated as nmol min^{-1} (mL accessible cellular space)$^{-1}$ using the aqueous cellular spaces accessible through the hepatic artery determined in a previous work [1]. These spaces are: 0.63 mL/g in anterograde perfusion (portal vein → hepatic vein), and 0.18 mL/g in retrograde perfusion (hepatic vein → portal vein).

aqueous cellular spaces that can be reached via the hepatic artery in anterograde and retrograde perfusion. These spaces were 0.63 mL/g for anterograde perfusion and 0.18 mL/g for retrograde perfusion [1]. The latter space consists solely of periportal cells [2, 3]. If the rates obtained in anterograde and retrograde perfusion are thus expressed as nmol min^{-1} (mL accessible cell space)$^{-1}$, they should reveal whether or not a given process predominates in periportal cells. Figure 2 revealed that, within the limits of the experimental errors, both curves do not differ from each other. Consequently, regarding their capacity of producing cAMP, the different liver cells that can be reached via the hepatic artery in anterograde and retrograde perfusion respond equally to glucagon.

Single Pass Extraction of cAMP

In the liver, cAMP can be hydrolyzed intra- and extracellularly [15, 16]. To verify if concentration gradients are generated when cAMP is infused, several experiments were performed in which single pass extraction was measured. In these experiments, cAMP was infused simultaneously with tracer amounts of [^{3}H]water. The latter was infused as an indicator for the recovery of the fluid pumped into the hepatic artery.

Table 1 shows the results found in this experimental series. The mean rates of cAMP infusion into the hepatic artery were approximately the same in both perfusion modes. Extraction of cAMP in both cases was not very pronounced. In anterograde perfusion, it was only 14%. In the retrograde mode, extraction was too small to be measured with confidence. From the results in Table 1 it can be concluded that concentration gradients along the sinusoidal beds are negligible.

Metabolic Effects of cAMP

To investigate the effects of cAMP on glycogen catabolism, cAMP was infused according to the four experimental protocols already employed for glucagon. The rate of cAMP infusion was the same in all experiments, namely 0.18 μmol min^{-1} g^{-1}, ensuring a final mean sinusoidal concentration of approximately 50 μM. The mean results of the experiments in which cAMP was infused into the hepatic artery are shown in Fig. 3. Qualitatively, cAMP exerted the same effects as glucagon [1, 17]: glucose release and oxygen uptake stimulation and glycolysis inhibition. In retrograde perfusion, however, the changes produced by cAMP were much smaller (Fig. 3B). This differs from the action of glucagon, which, as can be seen in Fig. 1, is practically independent of the infusion route and perfusion mode (see also Ref. 1). The metabolic effects were not increased by a 5-fold elevation of the infusion rate of cAMP (from 0.18 to 1.0 μmol min^{-1} g^{-1}; data not shown), indicating that 50 μM was already saturating.

Table 2 lists the changes found in the experiments illustrated by Fig. 3, together with the changes found when cAMP was infused into the portal vein (anterograde perfusion;

TABLE 1. Rates of infusion, recovery, and transformation of cAMP in the bivascularly perfused rat liver

	Inflowing cAMP	Outflowing cAMP	
Perfusion mode	(nmol min^{-1} g^{-1})		*P*
Anterograde (N = 4)	178.0 ± 7.9	152.9 ± 7.4	0.006
Retrograde (N = 4)	183.5 ± 27.4	181.3 ± 34.3	0.809

The bivascular liver perfusion was performed as described in Materials and Methods. cAMP was infused into the hepatic artery in either anterograde or retrograde perfusion. [^{3}H]Water was infused simultaneously as an indicator for the recovery of the fluid pumped into the hepatic artery. Samples of the inflowing and outflowing perfusate were collected for the measurement of [^{3}H]water and cAMP. Data are means ± SEM. Student's paired *t*-test was applied for analyzing the difference between inflowing and outflowing cAMP (*P* values).

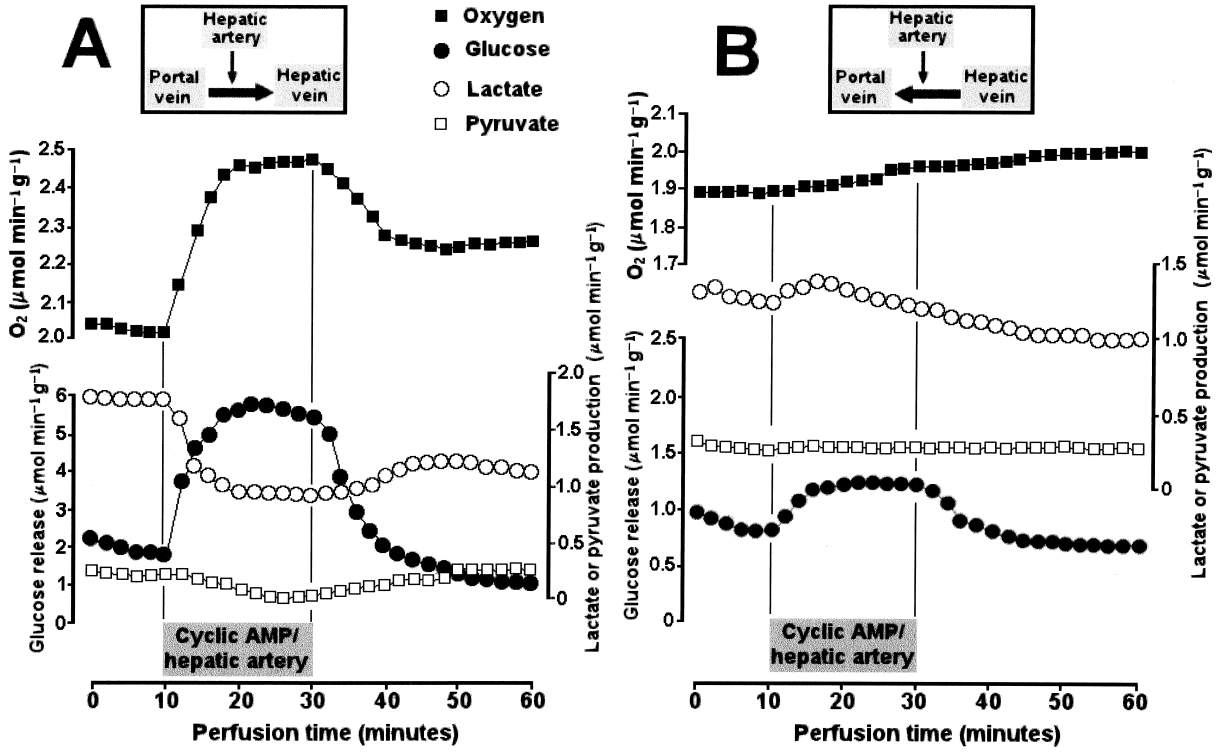

FIG. 3. Effects of cAMP infused into the hepatic artery on oxygen uptake and glycogen catabolism in rat livers perfused bivascularly in the anterograde (A) and retrograde (B) modes. Livers from fed rats were perfused as described in Materials and Methods. cAMP was infused into the hepatic artery at a rate of 0.18 μmol min^{-1} g^{-1} at 10–30 min. Samples of the effluent perfusate were taken for the measurement of glucose, lactate, and pyruvate. The venous oxygen concentration was measured polarographically. The data points are the means of 6 (A) or 8 (B) liver perfusion experiments using an identical protocol.

protocol C) and in the hepatic vein (retrograde perfusion; protocol D). A comparison of the rates expressed as μmol min^{-1} g^{-1} reveals that the action of cAMP on all variables was maximal when it was infused into the portal vein (anterograde mode) or into the hepatic vein (retrograde mode). This maximal action of cAMP was even superior to the action of glucagon. When infused into the hepatic artery in anterograde perfusion, the action of cAMP tended to be smaller, with the sole exception of glycolysis. The smallest action by far, however, was found in retrograde perfusion.

Table 3 allows us to compare the effects of cAMP infused into the hepatic artery after correction for the accessible cellular spaces [data as μmol · min^{-1} · (mL accessible cellular water space)$^{-1}$]. The retrograde to anterograde

TABLE 2. Increases in glucose release and oxygen consumption and decreases in lactate plus pyruvate production (glycolysis) due to cAMP (50 μM) in bivascularly perfused livers from fed rats

Perfusion mode and infusion route	Increase in glucose release	Increase in oxygen uptake	Decrease in glycolysis
	(μmol min^{-1} g^{-1})		
Anterograde perfusion and cAMP via the portal vein (N = 5)	4.58 ± 0.40	0.58 ± 0.10	0.95 ± 0.17
Anterograde perfusion and cAMP via the hepatic artery (N = 6)	3.63 ± 0.21†	0.45 ± 0.03	1.08 ± 0.16
Retrograde perfusion and cAMP via the hepatic vein (N = 3)	4.57 ± 0.21	0.58 ± 0.02	0.80 ± 0.22
Retrograde perfusion and cAMP via the hepatic artery (N = 8)	0.37 ± 0.10†	0.06 ± 0.02†	0.02 ± 0.07†

The bivascular liver perfusion was performed as described in Materials and Methods. The changes in glucose release, in oxygen consumption, and in lactate plus pyruvate production (glycolysis) were calculated by subtracting the basal rates (before cAMP infusion) from the rates found in the presence of cAMP. The metabolic fluxes are expressed as μmol min^{-1} (g liver wet weight)$^{-1}$ ± SEM.

† Statistically different from all other perfusion conditions according to the Student–Newman–Keuls test ($P < 0.05$).

TABLE 3. Metabolic changes per unit accessible cell space produced by cAMP and analogues infused into the hepatic artery in anterograde and retrograde perfusion

Agonist	Perfusion mode	Increase in glucose release	Increase in oxygen uptake	Decrease in glycolysis
		(μmol min^{-1} mL^{-1})		
cAMP	Anterograde (N = 6)	5.76 ± 0.33	0.71 ± 0.05	1.71 ± 0.25
	Retrograde (N = 8)	2.06 ± 0.55	0.34 ± 0.11	0.11 ± 0.39
	Retrograde/anterograde	0.36	0.48	0.06
		$P < 0.001$	$P = 0.018$	$P = 0.008$
N^6,2′-*O*-Dibutyryl cAMP	Anterograde (N = 4)	7.33 ± 0.98	0.62 ± 0.04	0.75 ± 0.13
	Retrograde (N = 5)	21.39 ± 2.61	2.56 ± 0.39	3.00 ± 0.31
	Retrograde/anterograde	2.92	4.13	4.0
		$P = 0.003$	$P = 0.003$	$P < 0.001$
N^6-Monobutyryl cAMP	Antrograde (N = 3)	4.77 ± 0.17	0.71 ± 0.79	1.16 ± 0.30
	Retrograde (N = 3)	19.89 ± 2.83	3.00 ± 0.61	4.72 ± 0.33
	Retrograde/anterograde	4.17	4.22	4.07
		$P = 0.006$	$P = 0.02$	$P = 0.001$

The changes in glucose release, in oxygen consumption, and in lactate plus pyruvate production (glycolysis) from Tables 2, 4 and 5 were recalculated as μmol min^{-1} (mL accessible cell space)$^{-1}$ employing the corresponding aqueous cell spaces that are accessible via the hepatic artery in each perfusion mode. These spaces were determined in a previous work and are 0.63 mL/g for anterograde perfusion and 0.18 mL/g for retrograde perfusion [1]. Values are means ± SEM. The *P* values were calculated by applying Student's *t*-test to each pair of values.

ratio was equal to 0.36 for glucose release (glycogenolysis), 0.48 for oxygen uptake, and 0.06 for glycolysis. Thus, in all cases, the effects of cAMP infused into the hepatic artery in retrograde perfusion were less than expected in regard to the accessible cell spaces.

Metabolic Effects of N^6,2′O-*Dibutyryl cAMP*

N^6,2′-*O*-Dibutyryl cAMP mimics quite well the effects of glucagon in several cell systems [18]. It is, thus, of interest to know if this effector behaves like cAMP or like glucagon when it is given to the 0.18 mL cells/g liver that can be reached via the hepatic artery in retrograde perfusion. For this reason, experiments were performed according to the same four protocols already employed in the case of cAMP. Figure 4 illustrates the mean results obtained when N^6,2′-*O*-dibutyryl cAMP was infused into the hepatic artery in anterograde (A) and retrograde (B) perfusion. The rate of infusion of N^6,2′-*O*-dibutyryl cAMP was 0.4 μmol min^{-1} g^{-1} (final concentration around 100 μM), which is saturating. As expected, N^6,2′-*O*-dibutyryl cAMP increased glucose release, decreased glycolysis, and stimulated oxygen uptake, basically the same effects as cAMP and glucagon. Quantitatively, the changes were similar to the maximal changes found with cAMP. However, unlike cAMP, the changes were also quite pronounced when N^6,2′-*O*-dibutyryl cAMP was infused into the hepatic artery in retrograde perfusion.

The mean changes produced by N^6,2′-*O*-dibutyryl cAMP in each of the four types of experiments are listed in Table 4. The increases in glucose release and oxygen consumption observed in the four perfusion modes were not significantly different. Differences appeared only in the case of glycolysis inhibition. But, even in this case, anterograde and retrograde infusion of N^6,2′-*O*-dibutyryl cAMP into the hepatic artery produced similar decreases.

The difference between cAMP and N^6,2′-*O*-dibutyryl cAMP becomes more evident if one compares the rates expressed as μmol min^{-1} (mL accessible cell space)$^{-1}$, which are shown in Table 3. In the case of N^6,2′-*O*-dibutyryl cAMP, the retrograde to anterograde ratio was equal to 2.92 for glucose release (glycogenolysis), 4.13 for oxygen uptake, and 4.0 for glycolysis. Thus, in all cases the effects of N^6,2′-*O*-dibutyryl cAMP, when infused into the hepatic artery in retrograde perfusion, were in excess of the accessible cell space.

Metabolic Effects of N^6*-Monobutyryl cAMP*

To see if it would be possible to reproduce the behaviour of N^6,2′-*O*-dibutyryl cAMP with another structural analogue, experiments were also performed with N^6-monobutyryl cAMP. Table 5 shows the mean changes, expressed as μmol min^{-1} (g liver)$^{-1}$, observed when this compound was infused into the hepatic artery via anterograde and retrograde perfusion. Table 5 reveals that the changes caused by N^6-monobutyryl cAMP were also practically independent of the perfusion mode. When expressed as μmol min^{-1} (mL accessible cell space)$^{-1}$, as shown in Table 3, the changes caused by N^6-monobutyryl cAMP in retrograde perfusion were also clearly in excess of the accessible cell space when compared with the effects in anterograde perfusion.

Kinetics of the Actions of Glucagon, cAMP, and its Structural Analogues

Table 6 lists the times required for half-maximal effects when the effectors used in this work were infused into the hepatic artery in anterograde and retrograde perfusion. Table 6 reveals that N^6-monobutyryl cAMP was the effector that required more time for the development of its

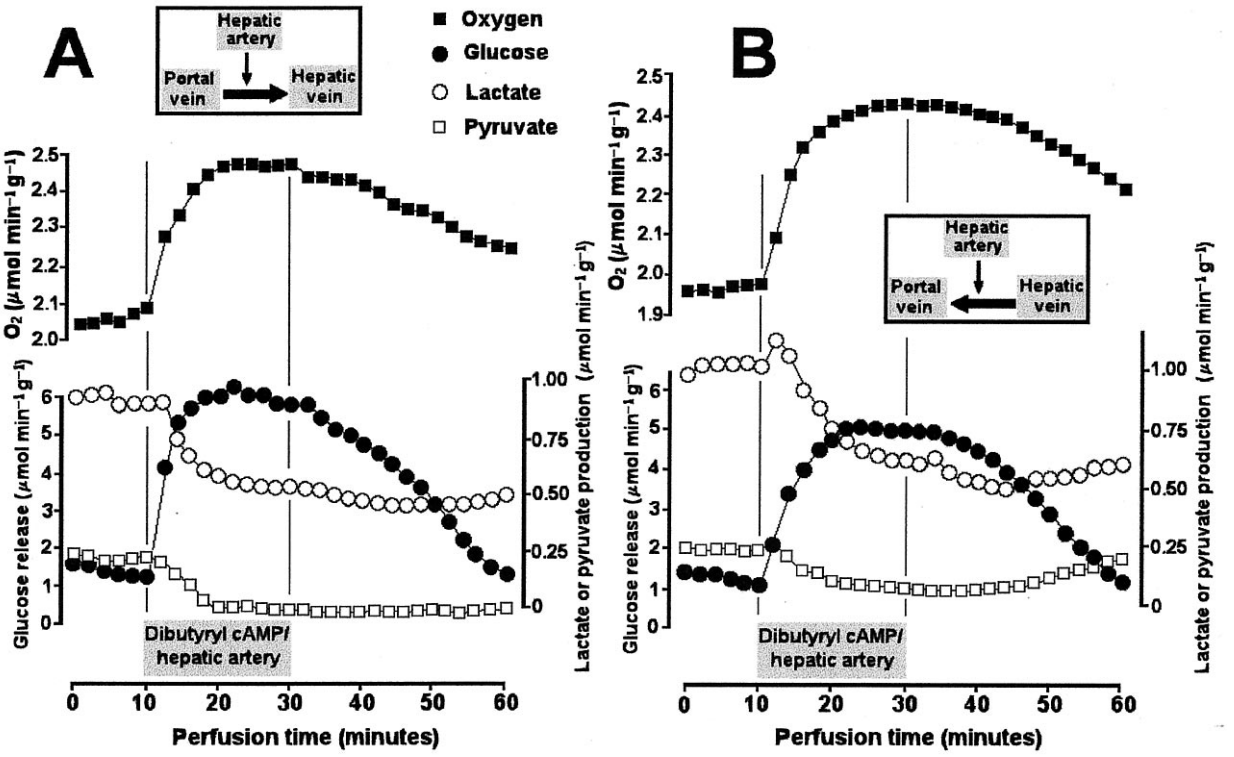

FIG. 4. Effects of N^6,2′-*O*-dibutyryl cAMP infused into the hepatic artery on oxygen uptake and glycogen catabolism in rat livers perfused bivascularly in the anterograde (A) and retrograde (B) modes. Livers from fed rats were perfused as described in Materials and Methods. N^6,2′-*O*-Dibutyryl cAMP was infused into the hepatic artery at a rate of 0.4 μmol min^{-1} g^{-1} at 10–30 min. Samples of the effluent perfusate were taken for the measurement of glucose, lactate, and pyruvate. The venous oxygen concentration was measured polarographically. The data points are the means of 4 (A) or 5 (B) liver perfusion experiments using an identical protocol.

effects. In anterograde perfusion it was the slowest one, while the others, cAMP, glucagon, and N^6,2′-*O*-dibutyryl cAMP, did not present significant differences. In retrograde perfusion, on the other hand, there was a general tendency for the effects to develop less rapidly, but the differences were significant for N^6,2′-*O*-dibutyryl cAMP and glucagon. Of the four agents, cAMP was the fastest one when infused into the hepatic artery in retrograde perfusion.

Reversal of glycogenolysis stimulation after stopping the infusion of each effector also presented significant differences. These differences are well illustrated by Fig. 5. In this figure, normalized rates of glucose release were represented against the time after cessation of the infusion of each effector. Normalization was accomplished by dividing the rates of glucose release at the different times after cessation of the infusion of each effector by the maximal steady-state rate of glucose release measured at the end of the infusion period. The times required for the decay in glucose release

TABLE 4. Increases in glucose release and oxygen consumption and decreases in lactate plus pyruvate production (glycolysis) due to N^6,2′-*O*-dibutyryl cAMP (100 μM) in bivascularly perfused livers from fed rats

Perfusion mode and infusion route	Increase in glucose release	Increase in oxygen uptake	Decrease in glycolysis
	(μmol min^{-1} g^{-1})		
Anterograde perfusion and N^6,2′-*O*-dibutyryl cAMP via the portal vein (N = 4)	4.10 ± 0.47	0.54 ± 0.04	1.05 ± 0.02*†
Anterograde perfusion and N^6,2′-*O*-dibutyryl cAMP via the hepatic artery (N = 4)	4.62 ± 0.62	0.39 ± 0.02	0.47 ± 0.08*
Retrograde perfusion and N^6,2′-*O*-dibutyryl cAMP via the hepatic vein (N = 4)	3.58 ± 0.18	0.42 ± 0.15	0.75 ± 0.17
Retrograde perfusion and N^6,2′-*O*-dibutyryl cAMP via the hepatic artery (N = 5)	3.85 ± 0.47	0.46 ± 0.07	0.54 ± 0.06†

The bivascular liver perfusion was performed as described in Materials and Methods. The changes in glucose release, in oxygen consumption, and in lactate plus pyruvate production (glycolysis) were calculated by subtracting the basal rates (before N^6,2′-*O*-dibutyryl cAMP infusion) from the rates found in the presence of N^6,2′-*O*-dibutyryl cAMP. The metabolic fluxes are expressed as μmol min^{-1} (g liver wet weight)$^{-1}$ ± SEM.

*† Pairs of values differing statistically according to the Student–Newman–Keuls test ($P < 0.05$).

TABLE 5. Metabolic effects of 100 μM N^6-monobutyryl cAMP infused into the hepatic artery

Perfusion mode	Increase in glucose release	Increase in oxygen uptake	Decrease in glycolysis
	(μmol min^{-1} g^{-1})		
Anterograde (N = 3)	3.01 ± 0.11	0.45 ± 0.05	0.73 ± 0.19
Retrograde (N = 3)	3.58 ± 0.51	0.54 ± 0.11	0.85 ± 0.06
	P = 0.336	P = 0.498	P = 0.579

The bivascular liver perfusion was performed as described in Materials and Methods. The changes in glucose release, in oxygen consumption, and in lactate plus pyruvate production (glycolysis) were calculated by subtracting the basal rates (before N^6-monobutyryl cAMP infusion) from the rates found in the presence of N^6-monobutyryl cAMP. The metabolic fluxes are expressed as μmol min^{-1} (g liver wet weight)$^{-1}$ ± SEM. The P values were determined using Student's t-test.

obeyed the following sequence: cAMP < glucagon < N^6,2′-O-dibutyryl cAMP ≈ N^6-monobutyryl cAMP. The difference between cAMP and its analogues was quite pronounced. In the case of N^6-monobutyryl cAMP, practically no decay was observed during the first 10 min. During the same time, the effects of cAMP had practically vanished. Figure 5 shows results obtained from experiments in which the livers were perfused in the anterograde mode and the effectors were infused into the hepatic artery, but a similar pattern was found for all other perfusion modes and infusion routes (not shown).

DISCUSSION

There are two sets of observations of this work that should be emphasized. The first is that stimulation of cAMP release caused by glucagon was proportional to the cellular spaces that were accessible via the hepatic artery in anterograde and retrograde perfusion and not to the metabolic effects of the hormone. This observation agrees with data concerning the distribution of the activities of glucagon-sensitive adenylate cyclase [19] and phosphodiesterase [20].

The second important observation is that, when corrected for the cell spaces that are accessible via the hepatic artery, the retrograde to anterograde ratios of the metabolic changes caused by glucagon [1], N^6,2′-O-dibutyryl cAMP, and N^6-monobutyryl cAMP were between 3 and 4, whereas for cAMP the corresponding ratios were between 0.1 and 0.5. This reflects the fact that cAMP behaved as a complete agonist only when it was supplied via the microcirculation and at saturating concentrations to all liver cells; by contrast, glucagon, N^6,2′-O-dibutyryl cAMP, and N^6-monobutyryl cAMP were full agonists even when they were given directly, at saturating concentrations, to the relatively small fraction of the liver cells that can be reached via the hepatic artery in retrograde perfusion (0.18 mL/g). Watanabe *et al.* [21] have measured the effects of glucagon infused during 5 min into the hepatic artery in anterograde and retrograde perfusion, at rates able to produce suboptimal concentrations (0.1 nM). Unfortunately, these authors did not analyze their data in terms of the accessible cell spaces. If one uses our cell space determinations, however, and transforms their data expressed as μmol per g into μmol (mL accessible cell space)$^{-1}$, one obtains a retrograde to anterograde ratio of approximately 2.2. Thus, the data of Watanabe *et al.* [21] also indicate metabolic effects of glucagon that are in excess of the accessible cell space. The disproportions found in our work were more pronounced, but the experimental conditions employed were not the same. In addition to the different hormone concentrations (saturating vs suboptimal) and the differences in quantitative analysis (areas under the curves vs final steady-state rates), the infusion times employed by Watanabe *et al.* [21] were only 5 min, a time that is not enough to reach

TABLE 6. Times for half-maximal stimulation of glucose release ($t_{max/2}$) after the onset of several effectors into the hepatic artery in anterograde perfusion

Effector	Time for half-maximal stimulation ($t_{max/2}$) (min)		P
	Anterograde perfusion	Retrograde perfusion	
cAMP	2.18 ± 0.19 (N = 6)	2.54 ± 0.45 (N = 8)	0.478
Glucagon	1.87 ± 0.14 (N = 6)	3.25 ± 0.30 (N = 9)	0.002
N^6,2′-O-Dibutyryl cAMP	1.82 ± 0.19 (N = 4)	3.70 ± 0.36 (N = 5)	0.004
N^6-Monobutyryl cAMP	2.83 ± 0.27 (N = 3)	4.05 ± 1.07 (N = 3)	0.331

Values were obtained from time–response curves of the kind shown in Figs. 3 and 4 by numerical interpolation using equation (1). Values are means ± SEM. The P values (Student's t-test) refer to a comparison between anterograde and retrograde perfusion.

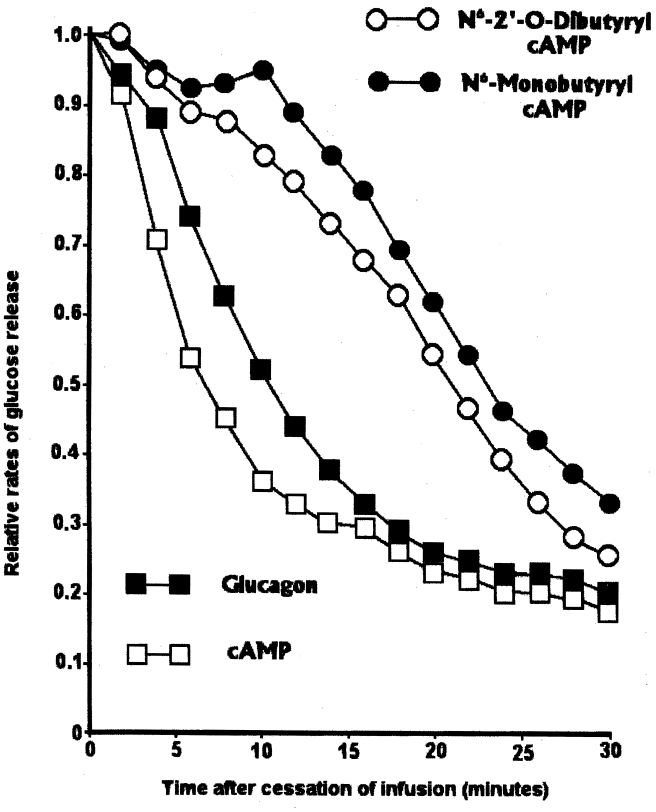

FIG. 5. Diminution of glucose release after stopping the infusion of glucagon, cAMP, N^6,2′-O-dibutyryl cAMP, and N^6-monobutyryl cAMP. The data were obtained from experiments in which the effectors were infused into the hepatic artery in anterograde perfusion. The relative rates of glucose release represent the rates after stopping the infusion of each effector divided by the rates immediately before. The data represent the means of 6 (cAMP), 3 (N^6-monobutyryl cAMP), 4 (N^6,2′-O-dibutyryl cAMP), and 6 (glucagon) liver perfusion experiments.

maximal effects even at saturating concentrations, as revealed by Table 6. Furthermore, as also shown in Table 6, the time for reaching the half-maximal effect in retrograde perfusion was considerably longer than in anterograde perfusion.

Lack of correlation between cAMP release and metabolic effects of glucagon in different liver cell populations has not been reported before. However, several authors have found disproportions between cAMP production or release and metabolic effects when glucagon concentrations were varied or when different effectors or hormones were introduced [4, 22–25]. By virtue of these findings, several authors have expressed the view that only a small pool of cAMP, produced under the stimulus of glucagon, is really active. This idea presupposes intracellular compartmentation of the active cAMP pool [22–24]. As an alternative, it has also been suggested that another intracellular effector (messenger), in addition to cAMP, could be acting in consequence of the glucagon stimulus [25–27]. The participation of Ca^{2+} has been particularly emphasized [26]. However, in our previous work we have found that the full response of glycogenolysis and oxygen uptake to glucagon when this agonist was infused into the hepatic artery in retrograde perfusion was not affected when Ca^{2+} was withdrawn from the system (medium and liver cells; [1]). Only glycolysis inhibition was affected, an observation suggesting that Ca^{2+} could be playing a secondary role, at least under the experimental conditions that were employed.

Taken as a whole, our results as well as several earlier observations that can be found in the pertinent literature, seem to conform much more with the much earlier hypothesis of a small active pool of cAMP [22–24]. The early finding of Exton and Park [28] that 60% of rat liver cAMP is associated with particles is consistent with this idea. cAMP would be able to leave this pool, and exogenously introduced cAMP would be almost excluded from it. Although the latter evidently has access to protein kinase A, endogenously generated cAMP could have a facilitated access to this enzyme, so that even small quantities would produce detectable metabolic changes. This view is corroborated by several observations. In our experiments, a much smaller amount of cAMP was produced when glucagon was infused into the hepatic artery in retrograde perfusion, but the metabolic effects were maximal. Exton *et al.* [4] and Okajima and Ui [22] found that glycogen decomposed without a detectable increase in the hepatic levels of cAMP when small doses of glucagon were given. The data of Corvera *et al.* [24] showing that the glucagon analogue [1-N^{α}-trinitro-phenyl-histidine,12-homoarginine]-glucagon was a full agonist for glycogenolysis stimulation, but an extremely weak agonist for cAMP accumulation, can also be explained in this way. The former hypothesis would be equally consistent with the fact that relatively high exogenous doses of cAMP are required in order to obtain metabolic effects that are comparable to those of glucagon. It is improbable that the passage of cAMP across the cell membrane, which seems to occur via a transport system [29, 30], was rate-limiting, because its effects developed as fast or even faster, depending on the perfusion conditions, as those of its analogue, N^6,2′-O-dibutyryl cAMP. It must also be remarked that when small quantities of cAMP were generated intracellularly by an alternative mechanism, as found by Yamatani *et al.* [23] after secretin infusion into the portal vein, no stimulation of glycogenolysis occurred. Yamatani *et al.* [23] also interpreted this finding as revealing compartmentation of intracellular cAMP production.

Besides favouring the access of cAMP to protein kinase A, compartmentation of this effector could also facilitate its transfer from one cell to another, as suggested by Sáez *et al.* [31] who found that glucagon and N^6,2′-O-dibutyryl cAMP are both able to increase junctional conductance. Transfer of cAMP from one cell to another would explain the observation that the responses of the liver to glucagon, when this hormone was given to all liver cells, was practically identical to the response when glucagon was given to only one-third or less of the liver cells, as happened when the hormone was infused into the hepatic artery via retrograde perfusion ([1] and Fig. 1). It should be noted that in the latter protocol, cAMP is probably present at saturat-

ing levels in the cells that can be reached directly via the microcirculation. In cells receiving the messenger by cell-to-cell diffusion, however, cAMP is more likely to be present at much lower concentrations. A cell-to-cell diffusion of cAMP would also explain the observation that cyanide, when introduced into the hepatic artery via retrograde perfusion at concentrations that completely block the mitochondrial respiratory chain, was not able to block oxygen uptake stimulation caused by glucagon, which was introduced simultaneously into the same vessel [1]. And finally, cell-to-cell diffusion would also be compatible with the finding that when glucagon was given directly to only one-third or less of the liver cells (via the hepatic artery in retrograde perfusion), it took more time to reach the full metabolic response than when it was given to a much greater fraction of the liver cells (anterograde perfusion; Table 6).

The hypothesis that glucagon-stimulated cAMP production is compartmentalized receives substantial support from the results obtained with the less polar cAMP analogues, N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP. The metabolic effects of these compounds, like those of glucagon, are also independent of the accessible cell spaces. Since N^6,2′-*O*-dibutyryl cAMP does not interact with protein kinase A [32–34], but its metabolic effects are quite pronounced, transformation within the cellular environment is essential. Transformation of N^6,2′-*O*-dibutyryl cAMP to cAMP can be catalyzed by non-specific deacylases (esterases), whose activity in the liver performs between 4 and 6% of the phosphodiesterase activity [35]. If one takes into account both the phosphodiesterase activity and the transport of cAMP to the outside of the cell, it is highly improbable that cAMP or even N^6-monobutyryl cAMP could accumulate to any considerable extent if transformation occurred in the aqueous cytosolic phase. Transformation of N^6,2′-*O*-dibutyryl cAMP within a nonpolar environment, on the other hand, seems the more likely alternative, a possibility that has already been raised by Blecher and Hunt [35]. The less hydrophilic nature of N^6,2′-*O*-dibutyryl cAMP should allow concentrative entry of this compound into nonpolar regions. The esterases are more likely to be found in the less polar regions of the cell, because their natural substrates are, most probably, nonpolar esters [35]. As in the case of the cAMP generated by virtue of the glucagon stimulus, cAMP generated within this environment could have a facilitated access to protein kinase A. The strongest indication that N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP are taken up in a concentrative manner by the liver is the fall-off kinetics of glycogenolysis after stopping the infusion of these effectors (Fig. 5). The observed difference between cAMP and its less polar analogues can be explained if one assumes that the latter have access, in a concentrative manner, to an extended intracellular space. If exogenously supplied cAMP and its analogues had access to identical cell spaces, their effects should decline at similar rates. Different rates of hydrolysis by the phosphodiesterase cannot explain the great differences revealed by Fig. 5. In the first place, the effects of both analogues, as discussed above, are actually the consequence of their transformation into cAMP. In the second place, Table 1 shows that the single pass extraction of cAMP was only 14%. Consequently, in an open system such as that employed in the present work, hydrolysis of cAMP is likely to play a minor role in the decay of the metabolic effects after cessation of the infusion. Moreover, the mean transit time of labelled water in the hemoglobin-free perfused rat liver is rarely superior to 17 sec [1]. A substance that distributes solely into the aqueous cell space is expected to be washed-off in less than 1 min, independently of being metabolized or not.

In conclusion, the results obtained in this work provide new evidence for the earlier hypothesis that cAMP, produced under the stimulus of glucagon, is compartmentalized in such a way that only small quantities are required for optimal metabolic effects [4, 22–24]. Possibly, cAMP production from N^6,2′-*O*-dibutyryl cAMP or N^6-monobutyryl cAMP occurs in the same compartment as the glucagon-stimulated cAMP production. Finally, a cell-to-cell diffusion of cAMP generated intracellularly, possibly via gap-junctions as suggested by Sáez *et al.* [31], is a strong possibility that deserves attention in future experimental work because it could be an explanation for the observation that glucagon, N^6,2′-*O*-dibutyryl cAMP, and N^6-monobutyryl cAMP are full agonists even when they are supplied to only 30% of the liver parenchyma.

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