BIOSYNTHESIS OF CYCLIC AMP ANTAGONIST IN HEPATOCYTES FROM RATS AFTER ADRENALIN- OR INSULIN-STIMULATION

Isolation, purification and prostaglandin E-requirement for its synthesis

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1. Introduction

Hormone-regulated and cAMP-mediated 'switch-on' of glycogen breakdown in liver or lipolysis in fat cells has been extensively studied. In contrast, little is known about the hormonal regulation of the 'switch-off' mechanism.

A feedback regulator in the incubation medium of fat cells was reported [1], which inhibited adenylate cyclase but activated cAMP-dependent protein kinase [2]. In [3,4] a second messenger for insulin was reported which should be a peptide of $M_r \sim 1500$.

In [5,6] the regulatory properties of a second messenger-type regulator were described, which inhibits adenylate cyclase and protein kinase and activates phosphoprotein phosphatase. Because of this counteraction to cAMP it was named cAMP antagonist. cAMP antagonist appears to be a mediator [7] of hormonally controlled switch-off mechanism in liver, muscle, heart and brain, since it inhibits the cAMP-dependent protein kinases in these tissues [5]. This report describes the biosynthesis and purification of cAMP antagonist.

2. Materials and methods

Male albino rats (Sprague-Dawley strain; 170–230 g body wt; fed ad libitum) were used. After anaesthesia with sodium nembutal (30–40 mg/kg body wt) livers were excised and hepatocytes were prepared as in [8]. Viability of cells as judged by trypan blue exclusion was always >80%. Adenylate cyclase activity was measured as in [9].

2.1. *Preparation and purification of cAMP antagonist*

cAMP antagonist was generated by incubating hepatocytes from 1 rat liver at 37°C in 30–50 ml gassed (95% O_2/5% CO_2) Krebs–Ringer bicarbonate buffer for 3–5 min with adrenalin (4.5×10^{-5} M) or insulin (5×10^{-8} M). The cells sedimented by centrifugation were resuspended in 30 ml 2.5 mM Tris–HCl buffer (pH 7.5) and homogenized with a Dounce. The homogenate was dialyzed 3 times against 150 ml 2.5 mM Tris–HCl buffer (pH 7.5) within 2 days, and after lyophilisation of the dialysis water cAMP antagonist containing raw material was obtained. Material from 4–6 rat livers was dissolved in water, poured over charcoal (cAMP antagonist does not adsorb), and was consecutively chromatographed on Sephadex G-15 (using 1.5 × 90 cm column and collecting 2 ml fractions, cAMP antagonist was eluted in fractions 42–48), QAE-Sephadex A-25 (using a 0.9 × 30 cm column and collecting 4 ml fractions, cAMP antagonist was eluted in fractions 18–20, when a linear gradient of 150 ml NaCl, 0–400 mM in 50 mM Tris–HCl buffer (pH 7.4) was applied), Sephadex LH-20 (using a 2.2 × 50 cm column and collecting 2 ml fractions cAMP antagonist eluted in fractions 59–64 ahead of NaCl, when elution was performed with 60% aqueous ethanol) and silicic acid (1.1 × 10 cm column equilibrated with benzene/ethylacetate/methanol at 60/40/10, and applying a gradient from 9–100% methanol cAMP antagonist was eluted at 50–60% methanol). The final step consisted of high-pressure liquid chromatography (HPLC) and was performed on a Nucleosil-dimethylamino column (Macherey and Nagel, Düren) equilibrated with 10 mM Tris–HCl buffer (pH 7.4);

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2 ml flow/min, and developed with a linear gradient from 0–500 mM NaCl on a 30 min programme run.

2.2. *Assay of cAMP antagonist*

cAMP antagonist activity was assayed by:

(i) Inhibition of cAMP-dependent protein kinase as in [5];

(ii) Its ability to inhibit adenylate cyclase.

Fractions of 10–50 μl containing cAMP antagonist were added to 600 μl of the adenylate cyclase assay mixture, when purified plasma membranes were used [9]. For measurements of adenylate cyclase activity in intact hepatocytes, the assay mixture contained $5–10 \times 10^6$ hepatocytes, 1 mM theophylline, 20 μM adrenalin and 200 μl of a fraction containing cAMP antagonist in a final volume of 1.5 ml gassed Krebs–Ringer bicarbonate buffer. Incubations were performed for 2 min at 37°C.

At present, the specific activity of cAMP antagonist cannot be given on a molar basis and is therefore expressed in units. One unit is defined as the amount of cAMP antagonist, which inhibits protein kinase by 50% under the experimental conditions described and in 0.1 ml reaction volume.

3. Results

Stimulation and restimulation of hepatocytes with glucagon each elicited cAMP synthesis. With adrenalin, however, no increase of cAMP synthesis was observed after a second stimulation with adrenalin [10], suggesting that adrenalin stimulation of hepatocytes leads to synthesis of an intracellular regulator, which inhibits adenylate cyclase.

3.1. *Isolation and purification of cAMP antagonist*

To demonstrate the existence of this hormone-stimulated regulator, adrenalin-stimulated and non-stimulated hepatocytes were homogenized (see section 2.1), the homogenates were centrifuged at 30 000 × *g* and the supernatants and resuspended sediments were dialyzed against water. Two adenylate cyclase inhibiting activities were present: one activity in form of a small diffusible molecule, which was more pronounced on hormonal stimulation, and the other activity as a non-diffusible, membrane-bound component of protein nature (it sedimented with the plasma membrane fraction in a sucrose gradient; it is denatured by heating 5 min at 80°C or by treatment with 100% ethanol).

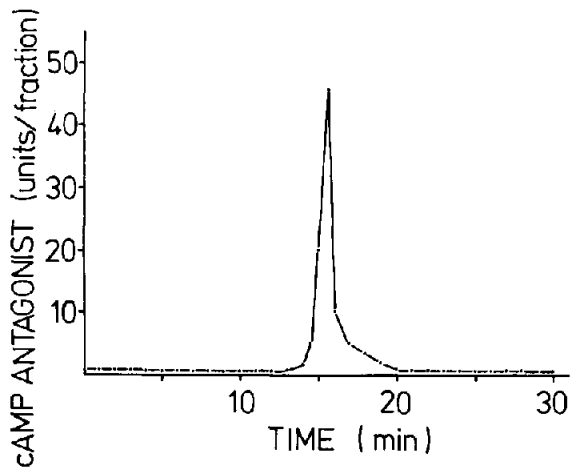

Fig.1. High-pressure liquid chromatography of cAMP antagonist on $N(CH_3)_2$-Nucleosil (10 μm); flow rate 2 ml/min; linear gradient (0–500 mM) NaCl in 10 mM Tris–HCl buffer (pH 7.4) on a 30 min programme run; 1 ml fractions were collected and assayed for cAMP antagonist.

The inhibitory activity present in the supernatant was dialysable. This activity, attributed to cAMP antagonist inhibited the cAMP-dependent protein kinase, whereas the inhibitory activity of the purified plasma membrane does not inhibit the protein kinase.

From Sephadex G-15 cAMP antagonist was eluted close to ATP, suggesting an app. $M_r \sim 500$. From QAE-Sephadex cAMP antagonist was eluted a few fractions ahead of cAMP, but much later than prostaglandin E_1. Fig.1 shows a high-pressure liquid chromatogram of cAMP antagonist purification. A 3000-fold purification was obtained with the first 3 chromatography steps (table 1).

Nearly equal amounts of cAMP antagonist are isolated per rat liver on stimulation with adrenalin

Table 1
Scheme for the purification of cAMP antagonist

	mg	units	units/mg
Crude extract	4200	n.d.	0.055
Gel filtration	400	230	0.575
QAE-Sephadex chromatography and desalting on LH-20	10	210	21
Silicic acid chromatography	app. 1	165	165

Values relate to the material obtained from 10 rat livers; n.d. not determined; the units of the crude extract were evaluated by assuming that no activity was lost during gel filtration

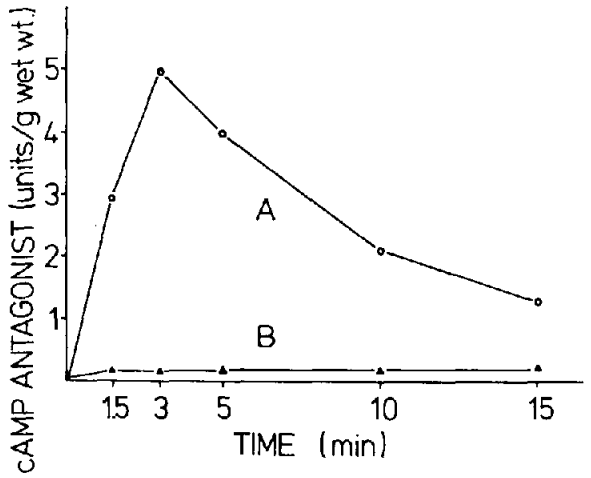

Fig.2. Time course of cAMP antagonist synthesis in hepatocytes on stimulation with adrenalin (4.5×10^{-5} M) (A) and without hormonal stimulation (B). (The shown experiment is representative for 5 separate expt.)

(22.5 ± 2 units) and insulin (19 ± 2 units). No detectable amounts of cAMP antagonist could be isolated from hepatocytes without hormonal stimulation. (Three separate experiments were performed, and in each experiment material of 10 rat livers was pooled.)

3.2. *Time course of cAMP antagonist synthesis*

Addition of adrenalin or insulin to hepatocytes immediately elicits cAMP antagonist synthesis, which culminates after 3–5 min and returns to basal level after 15–20 min (fig.2). When cAMP antagonist synthesis on adrenalin-stimulation was performed in presence or absence of adrenergic α- and β-receptor antagonists, results were obtained as shown in table 2. These data suggest that on adrenalin-stimulation cAMP antagonist synthesis is stimulated via adrenergic α-receptors just as cAMP synthesis is stimulated via adrenergic β-receptors.

3.3. *Regulatory properties of cAMP antagonist*

Equal amounts of cAMP antagonist per volume produce equal percentage of inhibition of both adenylate cyclase and protein kinase (fig.3). By definition 10 units of cAMP antagonist/ml assay volume inhibit these activities by 50% and 40–50 units/ml assay volume lead to 100% inhibition. The basal activity (without cAMP) and protein kinase fully activated by cAMP are inhibited by the same amount of cAMP antagonist to an equal extent. Inhibition of cAMP-dependent protein kinase by cAMP antagonist is non-competitive with respect to cAMP and ATP. The cAMP antagonist inhibits the separated catalytic subunit (not shown).

Table 2
Effects of α- and β-receptor blocking agents on adrenalin-stimulated cAMP antagonist synthesis

cAMP antagonist isolated on stimulation with	cAMP antagonist amount obtained	
	units	%
(1) Adrenalin	22.2	100
(2) Adrenalin plus propranolol	21	95
(3) Adrenalin plus phentolamine	9.9	45
(4) Control-incubation (without adrenalin)	0.9	4

cAMP antagonist was isolated after 4 min incubation and measured after purification by Sephadex G-15 chromatography using both adenylate cyclase assays (4.5×10^{-5} M adrenalin; 10^{-5} M β-receptor blocking agent, propranolol; 10^{-5} M α-receptor blocking agent, phentolamine. The shown experiment is representative for 3 separate expt.)

3.4. *Role of prostaglandin E in cAMP antagonist synthesis*

In [11] prostaglandin E (PGE) inhibited adenylate

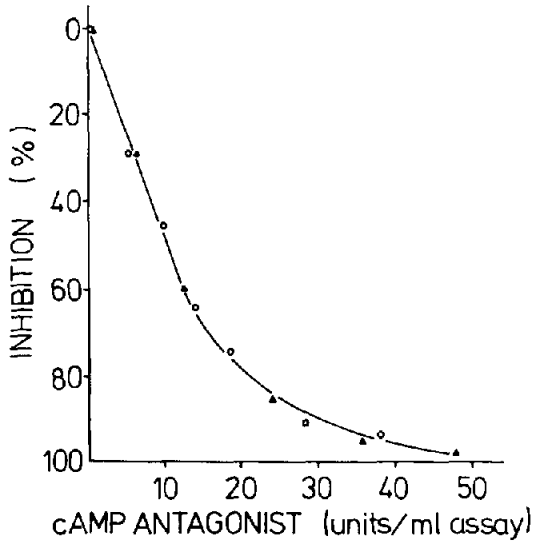

Fig.3. Concentration profile for the inhibition of adenylate cyclase (o——o) using plasma membrane assay and of protein kinase (▲——▲) by cAMP antagonist; 5, 10, 15, 20 and 25 μl aliquots of a solution of purified cAMP antagonist were added per 0.1 ml assay mixture as in section 2.

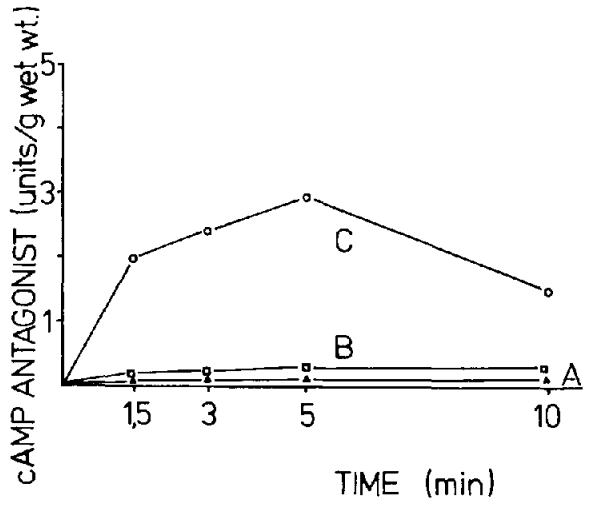

Fig.4. Time course of cAMP antagonist synthesis in hepatocytes of indomethacin-treated rats: (A) on stimulation with adrenalin (4.5×10^{-5} M); (B) in presence of 1.2×10^{-6} M PGE_1, but in absence of adrenalin; (C) in presence of PGE_1 on stimulation with adrenalin. (The shown experiment is representative for 3 separate expt.)

cyclase in intact fat cells. Similar effects of PGE can be shown in rat liver: by treatment of rats with indomethacin [12] (3–5 doses of 5 mg drug/kg body wt within 2 days i.p.) in a dose-dependent manner hepatocytes from such rats showed increased adrenalin- but not glucagon-stimulated cAMP synthesis (from 43 ± 5 pmol to 195 ± 45 pmol cAMP/5×10^6 hepatocytes; calculated from 7 expt). Essentially no cAMP antagonist could be isolated from these cells. Importantly, upon addition of PGE_1 to adrenalin-stimulated hepatocytes, cAMP antagonist synthesis was resumed (fig.4).

4. Discussion

The name cAMP antagonist used here for this new, intracellular, hormone messenger-like regulator merely denotes the fact that this substance acts in an antagonistic fashion to cAMP by inhibiting cAMP-dependent protein kinases and activating phosphoprotein phosphatases [5]. After stimulation of hepatocytes with insulin a substance can be isolated [7] with properties so far indistinguishable from that in adrenalin-stimulated hepatocytes, in that it behaves identically during chromatographic purification, and it inhibits protein kinase, activates phosphoprotein phosphatase [5,7] and activates pyruvate dehydrogenase [13]. These findings suggest that cAMP antagonist is an intracellular mediator of the action of a set of different (perhaps anabolic) hormones just as cAMP mediates the action of a number of different hormones.

Besides many different possibilities of controlling reduction of cAMP synthesis, attention was drawn to the fact that inhibition of adenylate cyclase is achieved by an adenylate cyclase inhibitor [1]. But the inhibitor isolated from fat cell media in [1], which activates protein kinase [2] is not identical with cAMP antagonist described here. As reported here, there is in addition to cAMP antagonist a membrane-bound inhibitory activity, which inhibits adenylate cyclase. The function of this inhibitor is presently unclear. In this context it is of interest to note that a cAMP-independent protein kinase activity is present in liver cell membranes, which can be activated by cAMP antagonist and which is already fully activated when hepatocytes have been stimulated with adrenalin prior to membrane isolation ([10], H. K. W. unpublished). It may be speculated that the adenylate cyclase is inhibited by cAMP antagonist via phosphorylation by this membrane-bound protein kinase after its activation by cAMP antagonist.

The observation [9] that liver plasma membranes with an adrenalin-responsive adenylate cyclase can be prepared after inhibition of prostaglandin synthesis by indomethacin supports the result in fig.4, suggesting that PGE is involved in the synthesis of cAMP antagonist. PGE_1 itself is not the cAMP antagonist, since PGE_1 neither inhibits adenylate cyclase of plasma membranes nor cAMP-dependent protein kinases. As will be shown in a following paper (in preparation) PGE_1 is a component of cAMP antagonist.

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