that the infusion vehicle itself was deciduogenic in rats, antagonistic effects between the deciduogenic stimuli, similar to those described in the present study, provide the most likely explanation for the inhibitory action of the ionophore in this case.

In conclusion, the present results strongly suggest that the polyphosphatidylinositol pathway in the hormonally-sensitized uterus of mice plays an important role in the transduction of deciduogenic signals from the lumen to the stroma to effect the DCR and the establishment of pregnancy. However, it appears that complex and sensitive arrangements between the second messenger molecules involve the pathway in this capacity, and further work is required to elucidate the precise mechanism of the process.

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Effect of monensin and diabetes on asialoglycoprotein degradation in rat hepatocytes

S. Gil-Falgon, J. Davy and J. Féger

Laboratoire de Biochimie, UFR des Sciences Pharmaceutiques et Biologiques, Université Paris-Sud, 5 rue J. B. Clément, F-92296 Châtenay-Malabry Cedex (France)
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Abstract. We have studied the effects of two modulations – streptozotocin-induced diabetes in vivo, and the presence of the carboxylic proton ionophore monensin in vitro – on the degradation of ^{3}H-asialoorosomucoid ligand in isolated rat hepatocytes.

The ligand was internalized by means of a synchronous wave procedure. Diabetes was associated with a marked decrease in the amount of total degraded radioactive ligand compared to that in normal cells (3.6% and 37.3% of internalized ligand respectively, at 60 min), together with increased secretion of degradation products into the incubation medium (87% and 46.3% of the total degraded ligand was secreted by diabetic and normal cells, respectively). Monensin induced similar effects in normal cells, but had no apparent effect in diabetic cells.
Key words. Hepatocytes; asialoglycoprotein receptor; degradation; diabetes; monensin.

Hepatic binding protein (HBP) is used as a receptor model for the investigation of the various steps of receptor-mediated endocytosis, i.e. internalization of the ligand-receptor complex, dissociation of the ligand from the receptor in acid endosomes, degradation of the ligand in acid lysosomes, and return of the receptor to the cell surface[1].

Monensin, a carboxylic ionophore which permits monovalent cation exchanges across cell membranes, inhibits acid-dependent ligand-receptor dissociation and ligand degradation[2], and has also been shown to reduce the number of HBP binding sites on the hepatocyte membrane[3].

We have previously shown that in rats with streptozotocin-induced diabetes the total uptake of asialoorosomucoid (ASOR) by hepatocytes is decreased due to a reduction in the number of cell-surface binding sites[4]. These observations raised the question as to whether modulation by monensin and diabetes mellitus might induce similar alterations in other steps of HBP endocytosis.

We therefore compared hepatocytes from normal and diabetic rats to examine a possible effect of monensin on ASOR degradation following a synchronous wave of ligand internalization.

Materials and methods

Human orosomucoid (Sigma) was desialylated with agarose-immobilized neuraminidase type X-A (EC 3.2.1.18) (Sigma), and ^{3}H-labelling was performed by reductive methylation according to Wilder et al.[5]. Specific radioactivity was 710 cpm/ng ASOR. Monensin, collagenase (type IV), bovine serum albumin and reagents for hepatocyte incubation (cell-culture-tested) were from SIGMA. $^3HKBH_4$ (50–65 Ci/mmol) was from CEA France. Male Sprague-Dawley rats (180–200 g) were obtained from Charles River breeders, France.

One group of animals received streptozotocin (65 mg/kg body weight, dissolved in isotonic saline and acidified to pH 4.5 with citric acid) via the tail vein. After 11 days, rats with blood glucose levels above 250 mg/ml were considered diabetic. Control rats were treated with excipient alone. Hepatocytes were isolated using the collagenase perfusion procedure of Berry and Friend[6]. Final cell pellets were suspended in Hepes buffer, pH 7.40 as described by Seglen[7], and contained 80–90% of single viable cells, as judged by 0.06% Trypan blue exclusion. Prior to use, the cells were incubated at 37 °C for 30 min to permit recovery of cryptic receptors[8]. Cells (3×10^6/ml in Hepes buffer) were allowed to bind ^{3}H-ASOR (2 µg/ml) at 4 °C for 60 min, washed three times to eliminate unbound ligand and then resuspended in prewarmed buffer at 37 °C in the presence or absence of 25 µM monensin.

At various incubation times an equal volume of ice-cold isotonic saline containing 20 mM EDTA was added and the cells incubated for 15 min at 4 °C. After three washes, the amount of intracellular ^{3}H-ASOR in the cell pellet was determined.

The rate of ^{3}H-ASOR degradation product secretion was measured at each time-point by mixing an aliquot of medium with an equal volume of ice-cold 2.5% phosphotungstic acid in 2 M HCl; after 15 min on ice, the precipitate was centrifuged at 700 × g for 10 min and radioactivity in the supernatant was determined.

A 20-min incubation at 4 °C with 0.055% digitonin under gentle agitation was used to permeabilize the cells and allow molecules above 200 kDa to diffuse out of the cells[9]. Centrifugation allowed the release of unbound undegraded and degraded ^{3}H-ASOR into the medium. As described above, a phosphotungstic acid (2.5% in 2 M HCl) treatment allowed the precipitation of undegraded ^{3}H-ASOR while degraded ^{3}H-ASOR was soluble[10]; radioactivity in both fractions was counted.

Non-specific radioactivity was usually determined by including 20 mM EDTA in analogous incubations. Similar results were obtained by incubations in the presence of 200 × unlabeled ASOR.

Statistical evaluations: The data presented in tables 1 and 2 are means ± SD. An analysis of variance (SYSTAT program) showed a highly significant difference for each parameter between normal and diabetic cells, with or without monensin. The one-sided paired-sample t-test was then used[11].

Results and discussion

Hepatocytes from normal and diabetic rats were incubated in the presence or absence of 25 µM monensin and 2 µg/ml ^{3}H-ASOR in such a way that a synchronous wave of internalization of radioactive ligand occurred.

As we reported in 1982[4], diabetes induced a decrease in the ligand binding capacity of HBP; the mean amount of specific prebound ^{3}H-ASOR was 17.6 ng/10^6 cells and 6.2 ng/10^6 cells from normal and diabetic rats respectively, very similar to the values previously observed[12].

Similarly, in this report we confirmed our earlier findings that hepatocytes from diabetic rats show a defect in the level of ligand internalization[12]: the maximum internalized ^{3}H-ASOR (tables 1 and 2) was 14.7 ng/10^6 cells and 3.9 ng/10^6 cells for normal and diabetic rat hepatocytes respectively, representing 83.5% and 62.9% of prebound ^{3}H-ASOR.

As monensin had no detectable effect on binding in normal or diabetic rats and induced only a slight effect on the level of internalization compared to controls in normal cells (70% compared to 83.7% respectively) (table 1), we suspected an effect on degradation of ^{3}H-ASOR. As shown in figure 1, the rate of degradation became linear in normal cells after about 30 min of incubation at 37 °C. In contrast, the amount of degradation products was markedly decreased in both diabetic and monensin-

Table 1. Fate of ^{3}H-ASOR in control and monensin-treated normal hepatocytes after 60-min incubation at 37 °C. Data are expressed as means ± SD obtained with duplicate determinations in four separate experiments. Statistical analyses were described in 'Materials and methods'. Significant differences vs control are marked by 2 and 3 asterisks for $p \leq 0.01$ and $p \leq 0.001$ respectively.

^{3}H-ASOR	Control	25 µM monensin
Internalized (ng/10^6 cells)***	14.7 ± 1.4	12.4 ± 1.09
Secreted degraded (ng/10^6 cells)***	2.51 ± 0.17	0.23 ± 0.03
Total degraded (ng/10^6 cells)***	5.5 ± 0.86	0.28 ± 0.04
Secreted degraded (%/max. internalized)***	17.2 ± 1.7	1.83 ± 0.15
Total degraded (%/max. internalized)***	37.3 ± 3.0	2.25 ± 0.13
$\%\left[\frac{\text{secreted degraded}}{\text{total degraded}}\right]$**	46.3 ± 6.2	82.3 ± 6.5

Table 2. Fate of ^{3}H-ASOR in control and monensin-treated diabetic hepatocytes after 60-min incubation at 37 °C. Data are expressed and were analyzed as in table 1. NS is non-significant.

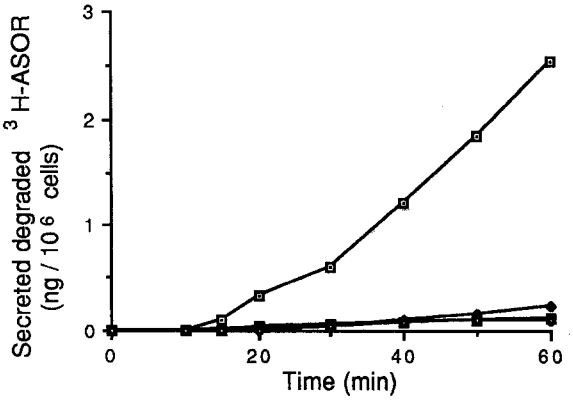
^{3}H-ASOR	Control	25 µM monensin
Internalized (ng/10^6 cells)^{NS}	3.9 ± 1.0	3.6 ± 0.6
Secreted degraded (ng/10^6 cells)^{NS}	0.12 ± 0.02	0.10 ± 0.01
Total degraded (ng/10^6 cells)^{NS}	0.14 ± 0.03	0.12 ± 0.01
Secreted degraded (%/max. internalized)^{NS}	3.11 ± 0.35	2.86 ± 0.62
Total degraded (%/max. internalized)^{NS}	3.62 ± 0.58	3.42 ± 0.82
$\%\left[\frac{\text{secreted degraded}}{\text{total degraded}}\right]^{NS}$	87.0 ± 9.1	83.9 ± 10.9

treated normal cells (0.10–0.23 ng/10^6 cells, respectively, at 60 min).

Degradative capacity of cells is usually evaluated by measuring acid-soluble radioactivity in the incubation medium, corresponding to secreted products. However, in order to determine whether diabetes and/or monensin might also affect the process of secretion/retention of degraded ligand, we measured total degraded products after permeabilizing the cells with 0.055% digitonin.

In normal hepatocytes, approximately 54% of degraded ligand was retained within the cells at 60 min (table 1). In contrast, monensin-treated cells, diabetic cells, and monensin-treated diabetic cells retained only about 18, 14, and 17% of degraded ligand respectively. The small amount of ligand which was degraded was almost entirely secreted by all three types of cell.

These results may reflect an innate capacity of cells to secrete a high proportion of degraded products rapidly when these are present in small amounts. Alternatively, it may indicate that all three types of hepatocytes have lost their retention capacity due to disorganization of



Effect of monensin on the degradation of ^{3}H-ASOR in hepatocytes from normal and diabetic rats.
We measured the secretion of degraded ligand by: (—□—) normal cells; (—◆—) monensin-treated cells; (—■—) diabetic cells and (—◇—) monensin-treated diabetic cells. Cells (3 × 10^6 cells/ml) were allowed to bind ^{3}H-ASOR (2 µg/ml, 4 °C, 60 min), washed three times with cold buffer and resuspended in duplicate with 37 °C prewarmed buffer in the presence or absence of 25 µM monensin for various times. Each point represents the mean of three separate experiments.

their intracellular membranes. This raises questions as to possible similar alterations of the same mechanisms by both modulations, i.e. monensin and diabetes. Monensin, which intercalates into membranes, alters pH and cation transmembrane gradients and causes a dramatic dilation and vacuolization of some Golgi cisternae [13, 14]. This rise in endosomal pH inhibits intracellular ligand dissociation [2] and, in this way, ligand degradation. An alteration of the acidification process may similarly take place in diabetes and prevent ligand dissociation and degradation. Indeed, in experimental diabetes, the known changes in membrane lipids may perturb the microenvironment of transmembrane active proteins such as the proton pump, giving rise to an alteration in intravesicular pH regulation [15, 16].

In addition, the disorganization of intracellular organelle membranes described in diabetic cells [17] may prevent the retention of the small amount of degraded products.

These mechanisms could explain why two such different modulators – a drug with precise effects and a disease involving complex hormonal malfunctions – give rise to apparently similar dysfunctions.

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Blockade of $GABA_B$ receptors accelerates amygdala kindling development

G. Karlsson, K. Klebs, T. Hafner, M. Schmutz and H. R. Olpe

Research and Development Department, Pharmaceuticals Division, Ciba-Geigy Ltd., CH-4002 Basel (Switzerland)
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Abstract. The aim of this study was to investigate the putative role of $GABA_B$ receptors in the development of amygdala kindling in rats. The effects of the $GABA_B$ blocker CGP 35348 and the $GABA_B$ agonist baclofen on the progressive development of behavioural seizure symptoms (stages 1–5 classified by Racine) and duration of afterdischarges (AD) were studied. CGP 35348 at a dose of 300 mg/kg i.p, which blocks central $GABA_B$ receptors, moderately but consistently accelerated the development of behavioural seizure symptoms. CGP 35348 had no marked effect on the duration of ADs corresponding to the different seizure stages. L-baclofen (6 mg/kg i.p.) had a dual effect on kindling development. It retarded the development of the behavioural symptoms, but increased the duration of AD. In conclusion, the results suggest that synaptically-released GABA activated $GABA_B$ receptors and thereby exerted a depressant effect on kindling development.
Key words. $GABA_B$ receptors; amygdala kindling; CGP 35348; baclofen; rat.

The roles of central gamma-aminobutyric acid ($GABA_B$) receptors are not well understood. The main source of information are in vitro studies demonstrating that presynaptically located $GABA_B$ receptors regulate the release of GABA [1]; postsynaptically they mediate a late inhibitory potential via activation of potassium channels [2, 3]. There is however a paucity of information on the functions of $GABA_B$ receptors in vivo. It is still unknown whether there is a '$GABA_B$ tone' under physiological conditions. It has been questioned recently whether endogenously released GABA activates pre- and/or postsynaptic $GABA_B$ receptors under physiological conditions [4]. In an attempt to obtain more insight into the function of $GABA_B$ receptors in vivo we investigated whether the blockade of $GABA_B$ receptors via the selective blocker CGP 35348 [5] or receptor stimulation with the $GABA_B$ agonist baclofen interfere with kindling development.

Kindling refers to the progressive development of behavioural and electroencephalographic epileptiform manifestations, triggered by repetitive delivery of an initially subconvulsive stimulus train [6]. The expression of epileptogenesis proceeds through characteristic behavioural stages which were classified by Racine [7]. Although the mechanisms that underlie the kindling process are not clear, the inhibitory neurotransmitter GABA appears to have an important role. It has been reported that drugs that augment GABA levels, such as GABA transaminase inhibitors (e.g. gamma-vinyl GABA [8]) and GABA uptake inhibitors (e.g. SK & F 89976-A [9]), may delay or block the kindling development in rats. Furthermore, drugs that increase GABAergic transmission by potentiating the effect of GABA at the postsynaptic $GABA_A$ receptor complex, e.g. benzodiazepines and phenobarbital [10], have also been shown to suppress kindling development. Conversely, drugs that decrease GABA levels by inhibition of GABA synthesis (3-mercaptopropionic acid) or block the postsynaptic $GABA_A$ receptor (bicuculline) have been reported to accelerate the kindling process [11].

CGP 35348 blocks pre- [12] and postsynaptic $GABA_B$ receptors [5] selectively up to a concentration of 1 mM. Since there are no pharmacological means available yet to affect pre- or postsynaptic $GABA_B$ receptors selectively, we chose rather high doses of the agonist and antagonist respectively which would presumably affect both types of receptors. The dose of baclofen could not be raised further because of its muscle-relaxing activity. The dose of CGP 35348 used has previously been shown to block central $GABA_B$ effects elicited by baclofen applied locally or systemically [5].

Materials and methods

Male rats (Tif: RAlf SPF) weighing 280–320 g at the time of surgery were anaesthetized with pentobarbital. Monopolar stainless steel depth electrodes were