Oxytocin inactivates and phosphorylates rat hepatocyte glycogen synthase

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Incubation of isolated rat hepatocytes with oxytocin produces a time- and dose-dependent inactivation of glycogen synthase. Such inactivation is associated with an increase in the phosphorylation state of the 88 kDa subunit of the enzyme, as observed after electrophoretic analysis of the ^{32}P -labelled enzyme isolated by immunoprecipitation from cells incubated with [^{32}P]phosphate. CNBr cleavage of the immunoprecipitated glycogen synthase showed that multiple sites were phosphorylated after exposure of the cells to the hormone. The effect of oxytocin on hepatocyte glycogen synthase activity was not observed in the absence of extracellular Ca^{2+} . Inactivation of glycogen synthase by oxytocin was partially abolished in the presence of insulin. These results indicate that the effects of oxytocin on glycogen synthase from rat hepatocytes are similar to those observed for other Ca^{2+} -mediated glycogenolytic hormones, such as vasopressin.

INTRODUCTION

The effects of oxytocin on glycogen metabolism have been studied in several tissues during the past few years. From studies using rat fat-cells, the existence of a dual mechanism for oxytocin action was proposed [1]. It was described that, at low concentrations (below 10 nM), the hormone showed a weak insulin-like activity on glycogen synthesis. On the contrary, at higher concentrations, oxytocin behaved as a glycogenolytic agent. This dual effect was explained by the existence of at least two types of receptors for oxytocin in rat adipocytes, as described by Bonne & Cohen [2].

More recently, Stephenson & Rogol [3] studied the effect of the hormone on glycogen synthesis and glycogen synthase activity in rat fat-cells. They found an inactivation of glycogen synthase after incubation with the hormone in a range of concentrations from 100 pM to 100 nM. In addition, the ability of oxytocin to inhibit the activation of glycogen synthase by insulin was reported.

The effect of oxytocin on hepatic glycogen metabolism was described by Whitton & Hems [4], who found an inhibition of glycogen synthesis. Furthermore, studies carried out with isolated hepatocytes demonstrated that oxytocin was able to produce an increase in glucose release and the activation of glycogen phosphorylase [5,6].

We decided to investigate the nature of oxytocin effects on hepatic glycogen synthase in order to compare its effects with those produced by other hormones and effectors. We have studied the effects of oxytocin on the activity and the phosphorylation state of this enzyme, which catalyses the rate-limiting step in glycogen synthesis. In addition, we have evaluated whether oxytocin and insulin behave in a synergistic or antagonistic way in rat hepatocytes with regard to their effects on glycogen synthase activity.

EXPERIMENTAL

Incubation of the cells

Isolated parenchymal liver cells were prepared from 24 h-starved Wistar rats (180–250 g) essentially as described in ref. [7]. Cells were resuspended in Krebs bicarbonate buffer (pH 7.4) free of glucose, pregassed with O_2/CO_2 (19:1). Portions [5 ml; $(6-8) \times 10^6$ cells/ml] were incubated at 37 °C with continuous shaking. At the end of the incubation with the effectors, cells were centrifuged (3000 g, 20 s) and cell pellets immediately homogenized with 300 μl of ice-cold 10 mM-Tris/HCl buffer (pH 7.4) containing 150 mM-KF, 15 mM-EDTA, 0.6 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine, 25 μg of leupeptin/ml and 50 mM- β -mercaptoethanol.

Radioisotopic labelling of rat hepatocyte glycogen synthase was performed by incubating the cells [5 ml; $(6-8) \times 10^6$ cells/ml] with [^{32}P]P_i (0.1 mCi/ml) in a low-phosphate (0.1 mM) Krebs bicarbonate buffer (pH 7.4) for 45 min. After this period, incubations with hormones were carried out and homogenates prepared as described above.

Assay methods

Homogenates were centrifuged at 10000 g for 20 min at 4 °C, and supernatants were assayed in duplicate for both glycogen synthase and phosphorylase activities. Glycogen synthase activity ratio was measured by the low (0.25 mM)-glucose 6-phosphate/high (10 mM)-glucose 6-phosphate method as described in ref. [8]. Glycogen phosphorylase activity was measured as in ref. [9], but with 1 mM-caffeine included in the mixture assay [10].

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Isolation and analysis of ^{32}P -labelled glycogen synthase

Homogenates obtained from ^{32}P -labelled cells were centrifuged at 10000 *g* for 20 min at 4 °C. Supernatants were ultracentrifuged (100000 *g* for 60 min at 4 °C) to obtain cytosolic supernatants containing glycogen synthase activity. ^{32}P -labelled glycogen synthase was immunoprecipitated from the 100000 *g* supernatants by incubation (2 h, 4 °C) with specific antibodies against rat liver glycogen synthase raised in rabbits [11].

Immunopellets were washed three times and dissolved in 200 μl of 50 mM- β -mercaptoethanol/70 % (v/v) formic acid. At this step, samples were taken to analyse the phosphorylation state of the enzyme subunit. The remaining sample was incubated overnight at room temperature with CNBr (12 mg/ml) in order to analyse the distribution of ^{32}P on the glycogen synthase molecule.

The phosphorylation state of both glycogen synthase subunit and CNBr fragments was analysed by SDS/polyacrylamide-gel electrophoresis. For that purpose samples were evaporated to dryness and resuspended in 20 μl of sample buffer, containing 40 mM-Tris/HCl (pH 6.5), 5 % (w/v) SDS, 0.6 M- β -mercaptoethanol, 20 % (w/v) sucrose and 0.0125 % Bromophenol Blue as a marker. SDS/polyacrylamide-gel electrophoresis was performed essentially as in ref. [12], in 6–12 %-acrylamide gradient gels. Autoradiograms were obtained by placing the dried gels at -80°C in X-ray cassettes containing films (Kodak X-Omat S) and intensifying screens. The amount of radioactivity was quantified by scanning the developed films at 546 nm.

Suppliers

$^{32}\text{P}]\text{P}_i$ was purchased from Amersham. Oxytocin and insulin were from Sigma. Chemicals for preparing cell media were from Merck. Other reagents were of analytical grade.

RESULTS

Effects of oxytocin on glycogen synthase activity

Incubation of rat hepatocytes for 8 min with different concentrations of oxytocin (1 pM–10 μM) resulted in a

dose-dependent inactivation of the enzyme, as measured by the low-/high-glucose 6-phosphate activity ratio assay (Fig. 1a). The maximal effect was obtained at 1 μM -oxytocin, and the half-maximal dose was about 0.1 μM . No change in glycogen synthase activity ratio was observed at concentrations below 10 nM as compared with control cells. Time-course experiments (Fig. 1b) demonstrated that, after addition of the hormone, a significant inactivation was obtained after 2 min. However, maximal effect was observed only after incubation for 8 min. Exposure of the cells for longer periods of time to oxytocin (up to 25 min) resulted in a partial restoration of activity. This behaviour was very similar to that observed for vasopressin (results not shown).

Oxytocin also provoked a noticeable activation of glycogen phosphorylase. Its activity increased from 80 ± 6 munits/mg in control cells to 241 ± 17 munits/mg in oxytocin-treated cells (mean $\pm$ S.E.M.). However, in this case the effects of the hormone were faster, the maximal activation being reached after incubation for 2 min at 1 μM (results not shown).

The effect of oxytocin on glycogen synthase was abolished in the absence of Ca^{2+} ions. Table 1 shows the results obtained after incubation of the cells with 1 μM -oxytocin for 8 min in a medium deprived of Ca^{2+} and supplemented with 1 mM-EGTA. As observed, no significant changes with respect to control cells were detected upon incubation with the hormone. Under the same conditions, the inactivation of glycogen synthase induced by glucagon was not affected by the absence of Ca^{2+} .

Effects of oxytocin on glycogen synthase phosphorylation state

Cells were labelled with $^{32}\text{P}]\text{P}_i$ and then incubated with 1 μM -oxytocin for 8 min. The enzyme was rapidly isolated by ultracentrifugation and immunoprecipitation techniques, and immunopellets were subjected to electrophoresis. These experiments demonstrated that oxytocin, at a concentration that provokes the inactivation of glycogen synthase, is also able to induce a clear increase (about 3-fold) in the amount of ^{32}P

Fig. 1. Inactivation of rat hepatocyte glycogen synthase by oxytocin

(a) Rat hepatocytes were incubated for 8 min with different concentrations of oxytocin (1 pM–10 μM), and glycogen synthase low-/high-glucose 6-phosphate activity ratio was determined. (b) Incubations were carried out for different times with 1 μM -oxytocin. Results are means $\pm$ S.E.M. for at least five experiments performed on different days.

Table 1. Ca^{2+} -dependence of the effects of oxytocin and glucagon on glycogen synthase activity

Cells were incubated either in normal Krebs–Ringer bicarbonate buffer (2.5 mM- CaCl_2) or in the same medium without Ca^{2+} and containing 1 mM-EGTA. Glycogen synthase activity ratio was measured as described in the Experimental section. Results are means $\pm$ S.E.M. of four independent experiments.

	Glycogen synthase activity ratio	
	+ CaCl_2	No CaCl_2
Control	0.39 ± 0.02	0.41 ± 0.03
Glucagon (10 nM)	0.26 ± 0.01	0.27 ± 0.02
Oxytocin (1 μM)	0.27 ± 0.02	0.39 ± 0.02

Table 2. Effect of oxytocin on the phosphorylation state of rat hepatocyte glycogen synthase

^{32}P -labelled immunoprecipitates were subjected to electrophoresis before and after CNBr treatment. Autoradiograms were scanned and the peaks integrated. Results are expressed as a percentage of the value of the peaks from control cells, and are means $\pm$ S.E.M. for at least five independent experiments. Data for glucagon are taken from ref. [20] and are included here for comparison.

	Glycogen synthase subunit	CNBr fragments		
		CB-2	CB-1	CB-2/CB-1
Control	100	100	100	3.1 ± 0.3
Oxytocin (1 μM)	272 ± 19	224 ± 22	347 ± 50	1.9 ± 0.4
Glucagon (10 nM)	191 ± 15	143 ± 11	260 ± 21	1.9 ± 0.1

Fig. 2. Electrophoretic analysis of glycogen synthase from ^{32}P -labelled cells

^{32}P -labelled glycogen synthase (GS) from control or oxytocin-treated cells was immunoprecipitated. Immunoprecipitates were subjected to electrophoresis before (a) or after (b) CNBr treatment: (1) control cells; (2) oxytocin-treated cells. Conditions are described in the text. Values for standard molecular-mass markers (kDa) are presented on the right.

covalently bound to the enzyme (Fig. 2 and Table 2). This increase is larger than that obtained after incubation of the cells with 0.1 μM -glucagon for 5 min, and very similar to that observed in cells incubated with 10 nM-angiotensin for 5 min (J. Ariño & J. J. Guinovart, unpublished work).

^{32}P -labelled immunoprecipitates from control and oxytocin-treated cells were cleaved with CNBr and subjected to electrophoresis. As expected [11,13,14], all the ^{32}P was found in two CNBr fragments, CB-2 and

Fig. 3. Counteraction by insulin of the effects of oxytocin on glycogen synthase activity

Cells were preincubated for 10 min in the presence of 0.1 μM -insulin (●) or in its absence (○), and then 0.5 μM -oxytocin was added and the incubation was continued for the indicated periods of time. Results are means $\pm$ S.E.M. for four independent experiments.

CB-1 (28 and 14 kDa respectively). Oxytocin was able to increase the amount of ^{32}P in both CB-2 and CB-1 fragments (Table 2). The increase in CB-2 was about 2-fold, whereas CB-1 showed a 3-fold increase.

Counteraction of the effects of oxytocin on glycogen synthase activity by insulin

Since these results indicate that in rat hepatocytes oxytocin is able to inactivate glycogen synthase, we studied whether insulin could affect the oxytocin effect

on glycogen synthase activity. For that purpose, cells were incubated for 10 min with $0.1 \mu\text{M}$ -insulin and then with $0.5 \mu\text{M}$ -oxytocin for different times. The results obtained (Fig. 3) indicated that insulin was able to counteract partially the inactivation of glycogen synthase provoked by oxytocin.

DISCUSSION

The results presented in this paper concerning the effect of oxytocin on glycogen synthase activity indicate that the hormone causes, at high concentrations, an inactivation of the enzyme, but never its activation. In this regard, oxytocin behaves like other glycogenolytic hormones, such as vasopressin and angiotensin II. Therefore, we have not been able to observe a biphasic effect of oxytocin on rat hepatocytes, such as that described in ref. [1] for adipocytes. However, it is noteworthy that the potency of oxytocin in inactivating glycogen synthase was at least 100-fold less than that of vasopressin and angiotensin II. The concentration of oxytocin able to produce a half-maximal effect is about $0.1 \mu\text{M}$, whereas this value is about 1 nM for the other hormones. This observation is in keeping with those by Hems *et al.* [5] and Kirk *et al.* [6], with regard to glucose mobilization and phosphorylase activation.

It has been described that the inactivation of glycogen synthase by hormones and other effectors in rat hepatocytes is accompanied by an increase in its phosphorylation state. Evidence has been presented for vasopressin and adrenaline [11,13], glucagon [13,15], phorbol esters [14] and vanadate [16,17]. As expected, a significant increase in the ^{32}P bound to the enzyme subunit was detected upon incubation with oxytocin, indicating that also in this case the inactivation of the enzyme is most probably the result of its phosphorylation. Since the increase in ^{32}P affected both CNBr fragments, it is concluded that oxytocin provokes the phosphorylation of glycogen synthase at least at two different sites. The changes in ^{32}P were quantitatively similar to those observed in our laboratory for vasopressin [11] and angiotensin (J. Ariño & J. J. Guinovart, unpublished work), although lower concentrations of these hormones were needed to obtain the same effect.

Regarding the mechanism by which oxytocin inactivates and phosphorylates glycogen synthase, a role for cyclic AMP-dependent protein kinase seems to be unlikely, since it has been reported that oxytocin does not change cyclic AMP levels in perfused rat liver [18]. On the other hand, an increase in phosphate incorporation into phosphatidylinositol in rat hepatocytes incubated with oxytocin has been reported [6]. Recently, Augert & Exton [19] reported that oxytocin caused an increase in 1,2-diacylglycerol, inositol phosphates and cytosolic Ca^{2+} in ghosts prepared from fat-cells previously labelled with [^3H]arachidonic acid. Our data, indicating that the effect of oxytocin on glycogen synthase activity is essentially lost when cells are incubated in a Ca^{2+} -depleted medium, support the idea that a Ca^{2+} -dependent mechanism is involved in the action of the hormone.

The effect of oxytocin in inactivating glycogen synthase was partially abolished by insulin. This result is in keeping with the observations by Stephenson & Rogol [3], who found a similar effect in rat fat-cells. In addition, we have observed that insulin is also able to counteract the effect of oxytocin in activating rat hepatocyte glycogen phosphorylase (results not shown). These results support the idea that oxytocin and insulin effects on hepatic glycogen metabolism are opposite and, as a consequence, that both hormones do not share a common post-receptor mechanism, as previously suggested [1].

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