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THE α-ADRENERGIC CONTROL OF RABBIT LIVER GLYCOGENOLYSIS

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Abstract—We have confirmed that the electrical stimulation of the splanchnic nerve in the rabbit causes glycogenolysis in a cyclic AMP-independent way as found by Shimazu and Amakawa [1]; glycogen phosphorylase (1,4-α-D-Glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1) was activated, but phosphorylase *b* kinase (ATP: phosphorylase *b* phosphotransferase, EC 2.7.1.38) was not. We could, however, not confirm the observation of a decrease in phosphorylase phosphatase (phosphorylase *a* phosphohydrolase, EC 3.1.3.17) activity. Pretreatment of the rabbits with the α-adrenergic blocking agent phentolamine prevented the splanchnic nerve stimulation from activating glycogen phosphorylase.

The addition of norepinephrine (10^{-7} M) to isolated rabbit hepatocytes activated glycogen phosphorylase without an activation of phosphorylase *b* kinase. At 10^{-6} M, norepinephrine activated both enzymes. Phentolamine blocked the activation of glycogen phosphorylase by norepinephrine at 10^{-7} M but not at 10^{-6} M. Absence of Ca^{2+} from the incubation medium prevented norepinephrine (10^{-7} M) from activating glycogen phosphorylase. The ionophore A 23187 also caused an activation of phosphorylase (but not of phosphorylase *b* kinase) provided that Ca^{2+} was present in the incubation medium. These data indicate that sympathetic nervous control of liver glycogenolysis is achieved, via α-adrenergic receptors, by an increased concentration of cytosolic Ca^{2+} ions which stimulate rather than activate phosphorylase *b* kinase. The neurotransmitter involved is most probably norepinephrine.

Shimazu and Amakawa [1] have shown that electrical stimulation of the splanchnic nerve in the rabbit leads to the activation of liver glycogen phosphorylase, the rate limiting enzyme in glycogen breakdown. The mechanism involved is not the cyclic AMP-initiated cascade of activations of protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37), phosphorylase *b* kinase and glycogen phosphorylase. Indeed, no increase in the concentration of the cyclic nucleotide was recorded and no activation of phosphorylase *b* kinase was induced by sympathetic nerve stimulation. Shimazu and Amakawa [1] have also observed a decrease in the activity of phosphorylase phosphatase, the enzyme responsible for the inactivation of glycogen phosphorylase. The intraportal injection of epinephrine produced the expected cyclic AMP-dependent activation of phosphorylase *b* kinase and phosphorylase, but no decrease in phosphorylase phosphatase activity. From their experiments Shimazu and Amakawa [1] suggested that the effect of sympathetic nerve stimulation might be mediated by release (or formation at the nerve terminals on liver cells) of some factor (called Factor X) other than norepinephrine.

An alternative explanation for these observations is that norepinephrine would be the neurotransmitter but would be acting as an α-adrenergic agent, using calcium ions and not cyclic AMP as the intracellular mediator. In recent years it has become progressively apparent that the stimulation of α-adrenergic receptors in liver cells leads to the activation of glycogen phosphorylase in a cyclic AMP-independent manner [2], probably by way of a calcium-dependent stimulation of phosphorylase *b* kinase [3–5] (We use the term 'activation' to designate a stable increase in the activity of an enzyme, such as that which occurs by phosphorylation of phosphorylase and phosphorylase *b* kinase; 'stimulation' designates an increased activity due to the freely reversible fixation of ligands such as calcium ions to phosphorylase *b* kinase which is a calcium-dependent enzyme; 'decrease in activity' means that the underlying mechanism is not clarified).

MATERIALS AND METHODS

Materials. Phosphorylase *a* was the third ethanol fraction obtained from dog liver [6] (8 U/mg protein) and was a gift of Dr. J. Goris and Dr. G. Defreyn. Crystalline rabbit muscle phosphorylase *b* was isolated according to Fischer and Krebs [7]. The heat stable protein kinase inhibitor was isolated up to the TCA precipitation step [8]: after dialysis against 5 mM potassium phosphate, pH 7.0, the preparation was clarified by centrifugation at 34,000 *g* for 20 min and further diluted as required with distilled water. The ionophore A 23187 was a gift of the Lilly Company (Indianapolis) through the courtesy of Lilly-Benelux (Brussels); the source of other chemicals has been given [4, 9].

Methods. The *in vivo* experiments were essentially done as described by Shimazu and Amakawa [1] but the liver samples were quick-frozen according to Wollenberger *et al.* [10].

Portions of the liver samples, kept at −20°, were cooled in liquid nitrogen and weighed when still frozen. Homogenization was done at 0° with concomitant

Abreviations: cAMP, cyclic AMP, adenosine 3′,5′-monophosphate; EGTA, ethyleneglycol-2-(2-aminoethyl)-tetraacetic acid.

thawing of the tissue. For the assay of phosphorylase *a*, we homogenized in 10 vol of an ice-cold medium containing 100 mM NaF, 20 mM EDTA, 0.5% glycogen and 100 mM glycylglycine, pH 7.4. For the assay of phosphorylase *b* kinase, the liver samples were homogenized in 10 vol. of the protein kinase inhibitor solution, at a concentration previously established to completely block protein kinase activity [11]. For the assay of phosphorylase phosphatase, we homogenized in 10 vol. of 10 mM Tris, 5 mM EDTA, pH 7.4; it was checked that this method yielded the same phosphatase activity as the homogenization technique of Shimazu and Amakawa [1]. Hepatocytes were isolated from young rabbits (500 g) whose livers were perfused through the vena cava posterior (right atrium); the cells were incubated and homogenized as described [11]. Succinctly, the cells (about 5×10^6 cells/ml) were suspended in the Krebs–Henseleit bicarbonate buffer gassed with 95% O_2/5% CO_2 and preincubated at 37° for 30 min in the presence of 20 mM glucose in order to bring phosphorylase *a* to a low level [12].

Phosphorylase *a* was measured as described in detail [12] except that the temperature was raised to 30°. For the assay of phosphorylase *b* kinase, 50 μl of the homogenate were mixed with 160 μl of a solution containing 0.4 mg of phosphorylase *b*, 60 mM NaF and 12.5 mM phosphate, pH 7.4. This mixture was then equilibrated at 30° for 2 min and the reaction was started by the addition of 20 μl of an ATP-Mg acetate solution, pH 7.4. The final concentrations of ATP and Mg were 1.3 mM and 9 mM respectively. The assay was then conducted as described [11]. One unit (U) of phosphorylase kinase catalyzes the formation of 1 unit of phosphorylase *a* per min under these conditions.

For the assay of phosphorylase phosphatase, 50 μl of the homogenate was added to 100 μl phosphorylase *a* solution (3 U) and 50 μl of a mixture containing 20 mM caffeine, 20 mM EDTA, 160 mM Tris, pH 7.4 [1]. After 0, 10 and 20 min at 30°, 50 μl were diluted with 0.7 ml of an ice-cold solution containing 55.5 mM NaF and 5 mM EDTA; 100 μl were used for phosphorylase *a* determination. One U of phosphorylase phosphatase catalyzes the inactivation of 1 U of phosphorylase *a* per min under these conditions.

RESULTS

Experiments with anesthetized rabbits. Electrical stimulation of the splanchnic nerve in adult rabbits produced within 30 sec an activation of liver glycogen phosphorylase which was maintained for at least 5 min. Mean values before and during nerve stimulation are given in Table 1: The activation of glycogen phosphorylase (+ 4.1 U/g liver) was not accompanied by an activation of phosphorylase *b* kinase or a decrease in the activity of phosphorylase phosphatase; no change in protein kinase activity was detected (not shown). An involvement of α-adrenergic receptors is indicated by a series of experiments using the α-adrenergic blocking agent phentolamine. Four rabbits received phentolamine (4 mg/kg) intravenously 5 min before the experimental procedure described in Table 1. Mean values of phosphorylase *a* (U/g liver) before and after stimulation were: 9.3 and 9.7; 10.3 and 10.3; 12.7 and 12.6; 11.3 and 11.1.

Experiments with isolated hepatocytes. The addition of norepinephrine (10^{-7} M) to isolated rabbit hepatocytes activated glycogen phosphorylase in the absence of a concomitant activation of phosphorylase *b* kinase. However, higher concentrations of norepinephrine (10^{-6} M) caused an activation of both glycogen phosphorylase and phosphorylase *b* kinase (Fig. 1). A similar activation of these enzymes was also obtained with glucagon (10^{-6} M), as was shown for rat hepatocytes [11]. The effect of norepinephrine (10^{-7} M) could be completely blocked by phentolamine (2×10^{-4} M); the effect of higher concentrations of norepinephrine (10^{-6} M) was hardly inhibited (Figs. 1 and 2).

The requirement for Ca^{2+} ions in the action of 10^{-7} M norepinephrine is demonstrated in Fig. 3: omission of Ca^{2+} ions from the incubation medium and addition of 2 mM EGTA prevented norepinephrine (10^{-7} M) from activating glycogen phosphorylase.

Table 1. Sympathetic stimulation of the rabbit liver *in vivo*

Experiment	Phosphorylase *a*			Phosphorylase *b* kinase			Phosphorylase phosphatase		
	U/g liver			U/g liver			U/g liver		
	before	after	effect	before	after	effect	before	after	effect
1	3.3	6.7	+ 3.4	9.3	8.6	− 0.7	—	—	—
2	7.3	12.1	+ 4.8	14.2	12.6	− 1.6	5.4	5.4	0.0
4	11.6	13.4	+ 1.8	8.0	6.8	− 1.2	7.9	7.9	0.0
4	12.3	16.1	+ 3.8	5.0	5.4	+ 0.4	—	—	—
5	12.3	17.1	+ 4.8	12.2	11.8	− 0.4	8.2	10.0	+ 1.8
6	18.9	24.9	+ 6.0	11.0	9.0	− 2.0	2.8	4.8	+ 1.0
Mean ± S.E.M.			+ 4.1* ± 0.6			− 0.9 ± 0.4			+ 0.7 ± 0.4

Four biopsies were taken from the livers of six anesthetized rabbits before sympathetic nerve stimulation (before) and four from 30 to 90 sec after the beginning of a continuous electrical stimulation (after). For each rabbit, mean values are given, and the effect is expressed by their difference. In view of the fairly large scatter of phosphorylase *a* values from one rabbit to the other, for which no explanation is readily available, statistical significance of the effect was calculated by the method of paired comparisons.

Significance is given as: * $P < 0.05$.

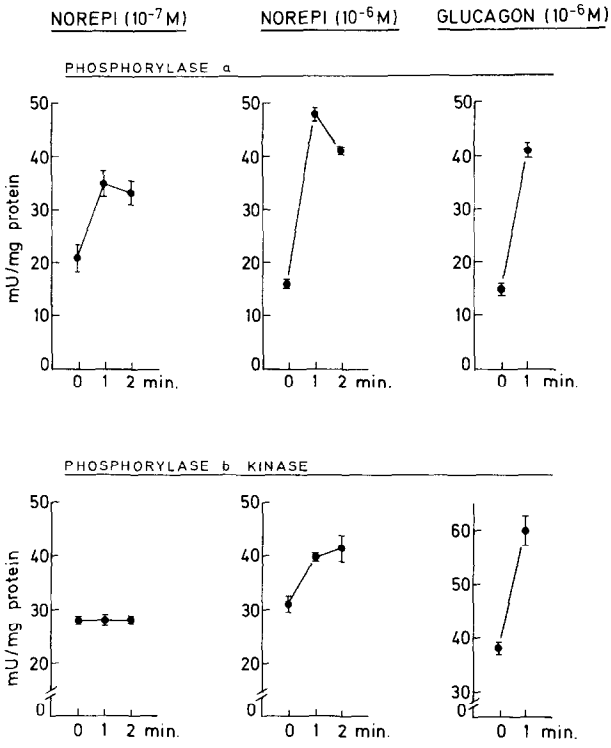

Fig. 1. The effect of norepinephrine and glucagon on glycogen phosphorylase and phosphorylase kinase. Rabbit liver cells were incubated at 37° (see Materials and Methods) and aliquots were taken immediately before and 1 and 2 min after the addition of the hormone. Values shown are means (± S.E.M.) for 3–6 experiments. It was verified that no activity changes occurred in the absence of norepinephrine (Norepi) and glucagon.

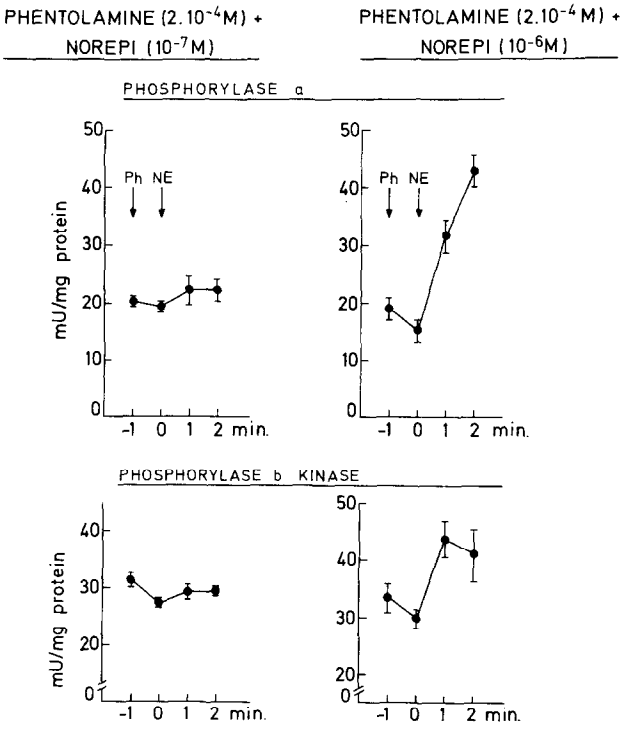

Fig. 2. Interference of phentolamine with the action of norepinephrine on phosphorylase and phosphorylase kinase. Phentolamine (Ph) was added 1 min before norepinephrine (NE). Values shown are means (± S.E.M.) for 3–6 experiments. Otherwise the same procedure is followed as in Fig. 2.

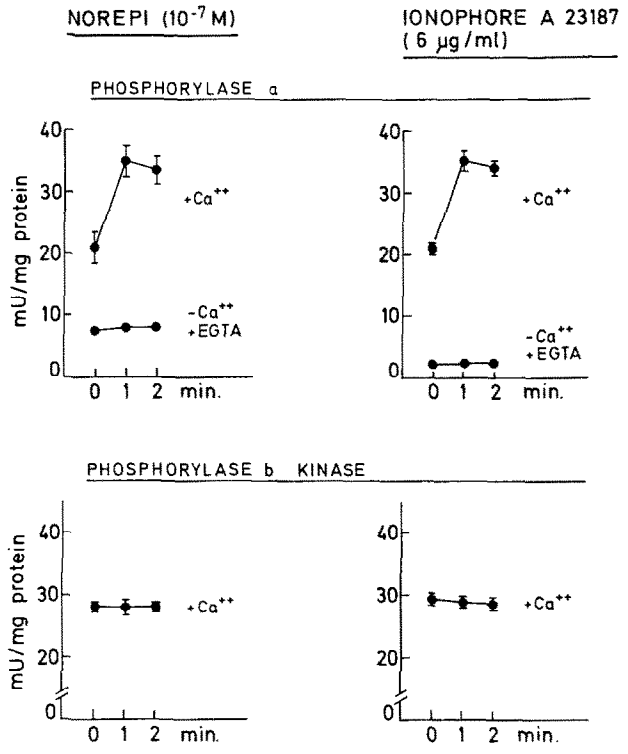

Fig. 3. The influence of the omission of calcium ions on the effect of norepinephrine and of the ionophore A 23187. Cells were incubated in the normal calcium containing medium (+ Ca^{2+}) or in a calcium-free medium containing 2 mM EGTA (– Ca^{2+}, + EGTA). The ionophore was dissolved in acetone; a control incubation with 0.5% acetone showed that the solvent by itself did not activate glycogen phosphorylase. Values shown are means (± S.E.M.) for 3–4 experiments except for the incubations without calcium where they are means of 2 experiments.

The Ca^{2+} ionophore A 23187, an agent specific for the translocation of divalent cations [13] led to an activation of glycogen phosphorylase provided that Ca^{2+} ions were present in the incubation medium; it was verified that no activation of phosphorylase *b* kinase occurred (Fig. 3). Other neurotransmitters and neuromodulators did not activate glycogen phosphorylase, at least when tested within 1 min after their addition (10^{-6} M) to the cell suspension. In duplicate experiments, the following levels of phosphorylase *a* (mU/mg protein) were recorded before and after the addition of dopamine (15.7 and 13.3), octopamine (12.6 and 11.1), tyramine (13.8 and 11.1), serotonine (13.5 and 10.5), Leu-enkephalin (13.9 and 14.9), Meth-enkephalin (15.4 and 12.3), substance P (13.8 and 11.0) and acetylcholine (14.4 and 14.3).

DISCUSSION

In a preliminary series of experiments (Table 1) we have partly confirmed the results of Shimazu and Amakawa [1]: electrical stimulation of the splanchnic nerve in the anesthetized rabbit elicits an activation of glycogen phosphorylase in the absence of an activation of protein kinase and of phosphorylase *b* kinase. The results are in agreement with their proposal that cyclic AMP is not involved. However, Shimazu and Amakawa [1] reported that splanchnic nerve stimulation produced a decrease in phosphorylase phosphatase activity; we could not confirm this observation, even although each rabbit served as its own control (Table 1).

From their experiments, Shimazu and Amakawa [1] concluded that the rise in hepatic phosphorylase *a* content upon splanchnic nerve stimulation is caused by a decrease in the activity of phosphorylase phosphatase rather than by a cyclic AMP-initiated acitivation of phosphorylase *b* kinase; they proposed the release of some new factor, other than norepinephrine. It should be noted however that in their experiments the decrease in the activity of phosphorylase phosphatase lagged behind the activation of glycogen phosphorylase: after 30 sec of splanchnic nerve stimulation, this decrease was far from complete (not more than 20 per cent inactivation) and still proceeded when the activation of glycogen phosphorylase had already reached a maximum level.

From our rather limited number of experiments we cannot confirm their observation of a decrease in the activity of phosphorylase phosphatase. On the other hand, we propose an alternative explanation (not necessarily mutually exclusive with that of Shimazu and Amakawa [1]) for the orthosympathetic activation of phosphorylase without invoking a new factor other than norepinephrine: the neurotransmitter involved is norepinephrine acting as an α-adrenergic agent, using

calcium ions and not cyclic AMP as its intracellular mediator. Indeed, an activation of rat liver glycogen phosphorylase, independent of cyclic AMP, has been documented for such glycogenolytic hormones as angiotensin and vasopressin and for α-adrenergic agents such as phenylephrine; all these agents probably use calcium instead as their intracellular mediator [3–5, 14], the target enzyme being phosphorylase *b* kinase which has been shown to be stimulated by micromolar concentrations of calcium ions [1, 5, 9, 15].

The fact that a pretreatment with phentolamine blocked the activation of glycogen phosphorylase by the splanchnic nerve stimulation clearly favors an involvement of α-adrenergic receptors. Furthermore, our experiments with isolated rabbit hepatocytes showed that at a concentration of 10^{-7} M norepinephrine activated glycogen phosphorylase in a manner compatible with the prevalent view of the mode of action of α-adrenergic agents: there was no activation of phosphorylase *b* kinase (Fig. 1), which suggests that cAMP was not the mediator; again, phentolamine prevented the activation of glycogen phosphorylase (Fig. 2). Calcium ions are presumably involved: (a) the use of a Ca^{2+}-free incubation medium prevented norepinephrine (10^{-7} M) from activating glycogen phosphorylase (Fig. 3); (b) the ionophore A 23187 led to an activation of that enzyme (also in the absence of an activation of phosphorylase *b* kinase) provided that Ca^{2+} was present in the incubation medium (Fig. 3).

Some caution is urged as to the origin of the increased cytosolic calcium. Although it has been implicitly admitted that the source of calcium ions is extracellular, this need not be the case. Indeed, from recent publications [16–18] it appears that an increased cytosolic calcium concentration may be due to the mobilization of intracellular Ca stores rather than to a net shift of calcium from the extracellular fluids to the cell. The inability of norepinephrine and ionophore A 23187 to activate glycogen phosphorylase in hepatocytes preincubated without calcium and with EGTA (see Fig. 3) should then be viewed as due to calcium depletion of intracellular reservoirs rather than to the absence of extracellular calcium ions *per se*. Indeed, stoichiometric chelation of extracellular calcium does not abolish the activation of phosphorylase by phenylephrine [18] and we have been able to confirm this observation for norepinephrine (10^{-7} M) which activated glycogen phosphorylase from 14.0 to 31.0 mU/g protein within 1 min (compare with Fig. 3).

We propose therefore that sympathetic nerve stimulation causes the release of norepinephrine, which will interact with α-adrenergic receptors and provoke the activation of liver glycogen phosphorylase by increasing the cytosolic calcium concentration. The target enzyme for the calcium ions is probably phosphorylase *b* kinase which has been shown to be a calcium-dependent enzyme [1, 15] with a K_A for calcium of 0.6 μM [5, 9, 15].

The activation of glycogen phosphorylase by norepinephrine at 10^{-6} M was always much higher than by norepinephrine at 10^{-7} M, was almost unaffected by phentolamine and was associated with an activation of phosphorylase *b* kinase (Figs. 1 and 2), indicating a cAMP-mediated β-adrenergic effect. The reason why splanchnic nerve stimulation does not lead to a β-mediated effect is most probably due to the fact that the concentration of norepinephrine in the vicinity of the receptors never reaches levels higher than those required for an α-effect. An alternative explanation would be that only α-adrenergic receptors can be reached by the neurotransmitter released from the synaptic endings. A series of neurotransmitters or neuromodulators tested in similar conditions as for norepinephrine did not display an effect on the glycogen phosphorylase which excludes them as possible candidates for a regulatory role in the control of liver glycogenolysis.

The proposal that norepinephrine is the neurotransmitter involved is in accordance with recent results of anatomical studies [19, 20] in which a noradrenergic innervation of hepatocytes of several species, including man [21], has been documented.

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