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Characteristics of the desensitization and resensitization of the cyclic AMP-independent glycogenolytic response in rat liver cells

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Vasopressin and α-adrenergic agonists were shown previously [Bréant, Keppens & De Wulf (1981) *Biochem. J.* **200**, 509–514] to induce a heterologous, dose-dependent and receptor-mediated desensitization of the cyclic AMP-independent glycogenolytic response in isolated hepatocytes. The desensitized state of the hepatocytes can be preserved as long as the agonist is bound to its receptor. Conversely, washing the cells with a hormone-free buffer or displacement of the agonist from its receptor by a specific antagonist restores the responsiveness. The desensitization and its reversibility (i.e. resensitization) are obtained within minutes. The desensitization can be clearly elicited at temperatures as low as 5°C, whereas the glycogenolytic response and the enhancement of the ^{45}Ca flux are only obtained above 15°C; the resensitization requires even higher temperatures. A tentative model is proposed to account for the observed effects.

Apart from the well-established cyclic AMP-dependent regulation of liver glycogenolysis, there is also a substantial cyclic AMP-independent glycogenolysis initiated by α-adrenergic agonists, vasopressin and angiotensin. It is currently admitted that the mechanism involved is an increase in cytoplasmic Ca^{2+}, which will stimulate phosphorylase kinase and so lead to the activation of glycogen phosphorylase, the rate-limiting enzyme in glycogen breakdown (see Rasmussen & Waisman, 1981). We (Bréant *et al.*, 1981) have shown that vasopressin and α-adrenergic agonists induce a dose-dependent desensitization of this cyclic AMP-independent glycogenolytic response in isolated rat liver cells. The same α-adrenergic or vasopressin receptors are involved both in the activation of glycogen phosphorylase and in the induction of the refractoriness, which has in addition clearly shown to be heterologous in nature. This latter property implies that the tachyphylaxis is due to the 'inactivation' of a common effector system beyond the agonist-receptor interaction.

We have now studied the kinetics and the temperature-dependency of the desensitization. The data show that it is induced within minutes and that it can also be reversed within minutes upon dissociation of the desensitizing agent from its receptor. The desensitization and resensitization can be disconnected from the other metabolic effects studied (phosphorylase activation and Ca^{2+} movements), since they occur at different temperatures. A model is presented to account both for the kinetics and the heterologous nature of this desensitization.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Experimental

Methods

We have used male Wistar-strain albino rats (200–250g body wt.), which were fed *ad libitum*. Liver cells were prepared as described previously (Vandenheede *et al.*, 1976) and incubated either in Krebs–Henseleit bicarbonate buffer equilibrated with O_2/CO_2 (19:1) (Krebs & Henseleit, 1932) or, for the experiments with La^{3+}, in a Hepes-buffered saline solution. This medium contains 119.4mM-NaCl, 4.8mM-KCl, 2.56mM-$CaCl_2$, 1.2mM-$MgCl_2$ and 20mM-Hepes, pH 7.4; phosphate and sulphate were omitted because of the poor solubility of their lanthanum salts. The cells (approx. 5×10^6 cells/ml) suspended in one of the two media were pre-incubated for 20–30min with 10mM-glucose and 14mM-bacitracin at 37°C in closed (Krebs–Henseleit) or open (Hepes) plastic 20ml vials with continuous shaking (120 oscillations/min). The study of the temperature-dependency required specific conditions as outlined in the legend to Fig. 7. The incubations with the α-adrenergic agents were done in the presence of stabilizing agents (3mM-catechol and 1mM-ascorbate) and of the β-

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adrenergic antagonist propranolol (0.1mM). The sampling procedure and assay of phosphorylase *a* activities were as described by Vandenheede *et al.* (1976). [^{3}H]Vasopressin binding was studied as described previously (Cantau *et al.*, 1980). The $^{45}Ca^{2+}$ fluxes were evaluated as described by Keppens *et al.* (1977). Succinctly, the uptake of $^{45}Ca^{2+}$ was monitored over a period of 6min by rapidly filtering cell samples at various time intervals after the addition of the tracer. Since non-steady-state conditions were used, these fluxes are not to be interpreted as net uptake, but merely indicate changes in the transmembrane Ca^{2+} movements. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. When data from single experiments are shown, these have been shown to be representative for several similar experiments.

Chemicals

[Arg^8]Vasopressin (grade VI), phenylephrine, propranolol and catechol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Angiotensin was from Schwarz–Mann, Orangeburg, NY, U.S.A. $^{45}CaCl_2$ was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). [*Tyr*^{2}-^{3}H][Lys^8]Vasopressin was a gift from Professor S. Jard (Collège de France, Paris, France).

Results and discussion

To study the reversibility of the desensitization, it is mandatory to dissociate the agonist from its receptor. This can be achieved intentionally by washing the cells free of the agonist, by applying an appropriate antagonist (see below), or incidentally by spontaneous degradation of the desensitizing agent. We have observed this last phenomenon for angiotensin (Bréant *et al.*, 1981), and it most probably accounts for the failure of some groups (Aggerbeck *et al.*, 1980; Blackmore *et al.*, 1982) to obtain an α-adrenergic desensitization, since their experiments were performed in the absence of protective agents such as ascorbate and catechol.

Washing of cells previously desensitized by vasopressin results in a restoration of their sensitivity to a second challenge by vasopressin, unless this washing is done in the presence of desensitizing concentrations of the hormone (Fig. 1). Similar results have been obtained with phenylephrine (results not shown). Dissociation of an agonist from its receptor can also be induced by a specific antagonist. The use of the readily available α-adrenergic antagonist phentolamine is appropriate for the study of the reversibility of the α-adrenergic desensitization. Antagonistic analogues of vasopressin, however, are not currently available at the required amounts. In a search for another agent to

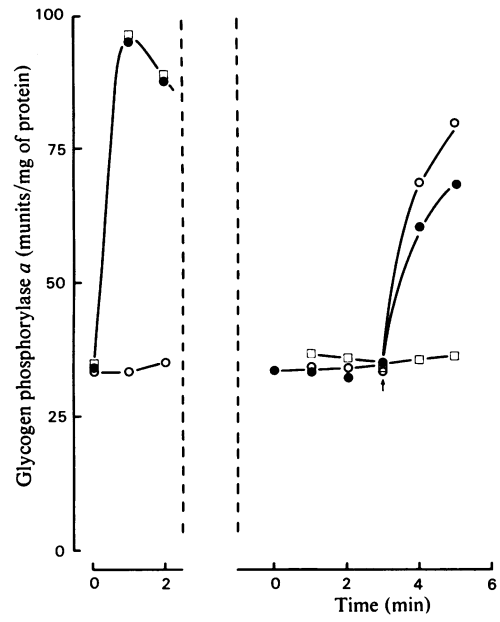


Fig. 1. *Reversibility of the vasopressin-induced desensitization by washing*

Preincubated hepatocytes (2ml) were further incubated with (●, □) or without (○) 25nM-vasopressin for 2min; they were then washed twice at 20°C by centrifugation at 50*g* for 30s in 50vol. of fresh Krebs–Henseleit bicarbonate buffer in the presence (□) or absence (○, ●) of 25nM-vasopressin. The final pellets were taken up in 1ml of the same buffer (with or without vasopressin) and again incubated at 37°C for 3min. The cells were then challenged with 25nM-vasopressin (arrow) and the changes in phosphorylase *a* levels were determined. The washing procedure was done within 4min and at least 85% of the remaining cells excluded Trypan Blue.

displace vasopressin from its hepatic receptors, we came across the property of La^{3+} ions to fulfil such a role. The ability of $LaCl_3$ to displace [^{3}H]-vasopressin from its receptor is shown in Fig. 2, which shows that the salt is as effective as an excess of unlabelled vasopressin to cause the dissociation of the radioligand from hepatocytes. Conversely, 1mM-$LaCl_3$ completely blocks the dose-dependent activation of glycogen phosphorylase by vasopressin, but does not interfere with the effect of angiotensin (Fig. 3). This property allows us to study the reversibility of the vasopressin-induced desensitization by using $LaCl_3$, which will not at all prevent a subsequent angiotensin-dependent activation of phosphorylase. It is noteworthy that angiotensin can activate phosphorylase in the presence of 1mM-La^{3+}, since this ion is frequently

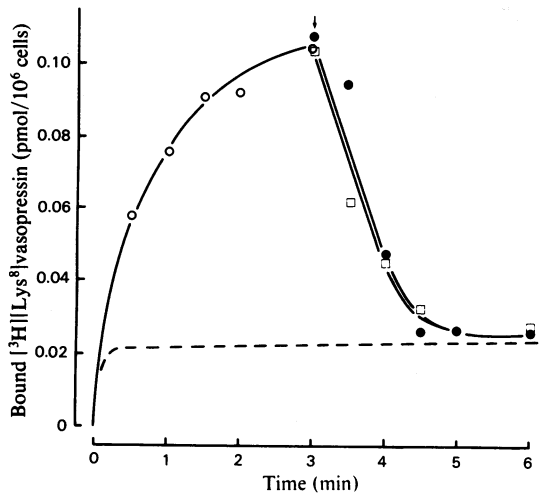


Fig. 2. *Binding of [^{3}H][Lys^8]vasopressin to hepatocytes and its dissociation by $LaCl_3$*

The association–dissociation study was done as described by Cantau *et al.* (1980), except that an appropriate Hepes-buffered saline (see under 'Methods') was used. At 3 min after the addition of 10 nM-[^{3}H][Lys^8]vasopressin to a cell suspension (10^6 cells/ml), either 1 mM-$LaCl_3$ (□) or 100-fold excess of vasopressin (●) was added. Non-specific binding is indicated by the broken line.

used to inhibit Ca^{2+}-mediated responses in various tissues.

Figs. 4 and 5 illustrate both the rapidity of onset and of reversibility of the desensitization by phenylephrine and by vasopressin. Fig. 4 shows that 2 min of α-adrenergic stimulation renders the cells completely resistant to vasopressin (the resistance to vasopressin as early as 2 min is complete, since phosphorylase activity declines as rapidly as without vasopressin); sensitivity can be partially reinstated as early as 2 min after the addition of the α-adrenergic antagonist phentolamine and is essentially complete within 10 min. Similar results for the vasopressin desensitization are presented in Fig. 5, where $LaCl_3$ was used to dissociate vasopressin from its receptors (see Fig. 2) and angiotensin was used as the second stimulus of phosphorylase activation. Since Figs. 4 and 5 illustrate results from single experiments, no particular attention should be paid to the slight kinetic differences.

From other studies it was known that the enhanced Ca^{2+} fluxes caused by α-adrenergic agonists are as transient as the activation of glycogen phosphorylase (Blackmore *et al.*, 1979; Murphy *et al.*, 1980). We have confirmed these observations and found that the cells can also be rendered refractory in so far as Ca^{2+} movements are

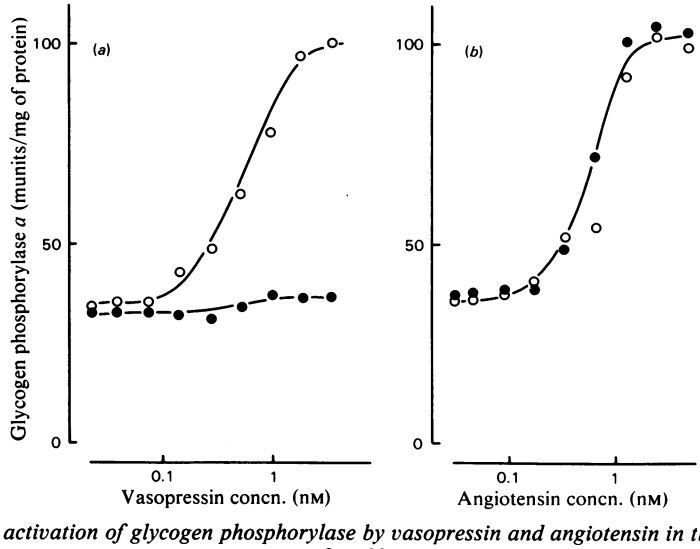


Fig. 3. *Dose-dependent activation of glycogen phosphorylase by vasopressin and angiotensin in the presence and absence of $LaCl_3$*

Hepatocytes were incubated with increasing amounts of vasopressin (*a*) or of angiotensin (*b*) in the absence (○) or presence (●) of 1 mM-$LaCl_3$ added 1 min earlier. Phosphorylase was assayed 1 min after the addition of the hormones. The sensitivity of the cells to the hormones in this particular Hepes buffer (see under 'Methods') is slightly less than previously obtained in the Krebs–Henseleit bicarbonate medium (Bréant *et al.*, 1981; Keppens & De Wulf, 1976).

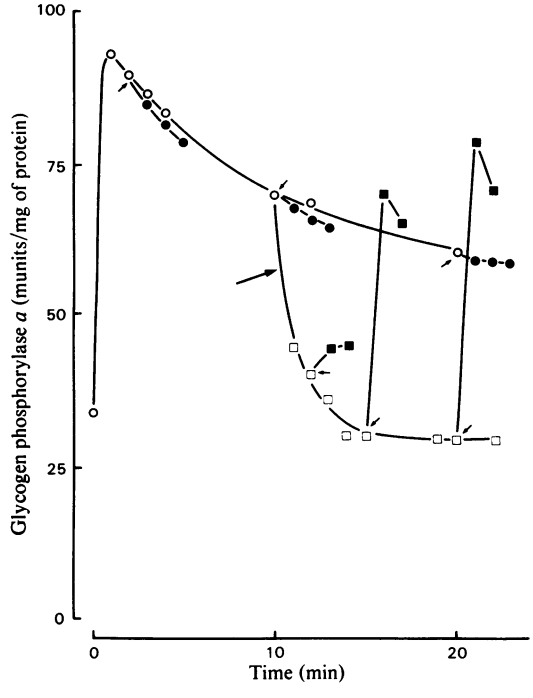


Fig. 4. *Kinetics of the phenylephrine-induced desensitization*

Several cell suspensions were treated with 10 μM-phenylephrine. One was incubated without further additions (○). Others were treated at the times indicated (short arrows) with 25 nM-vasopressin in the absence (●) or in the presence (■) of 0.1 mM-phentolamine (added at 10 min, large arrow), whose influence was also monitored (□).

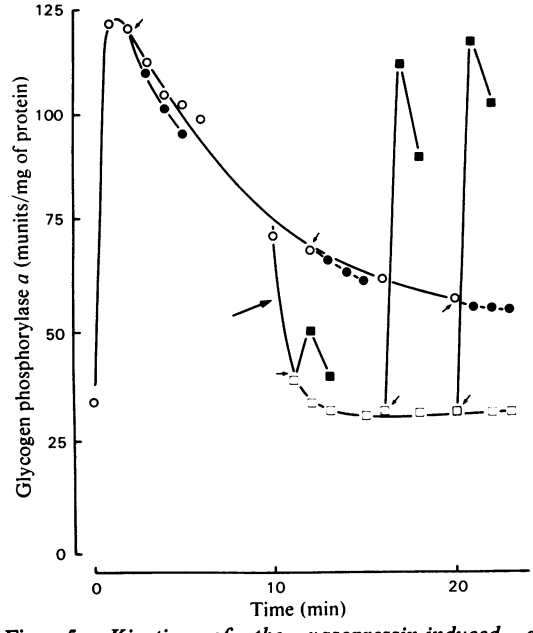


Fig. 5. *Kinetics of the vasopressin-induced desensitization*

Several cell suspensions incubated in the saline–Hepes solution (see under 'Methods') were treated with 25 nM-vasopressin. One was incubated without further additions (○). Others were treated at the times indicated (short arrows) with 40 nM-angiotensin in the absence (●) or in the presence (■) of 1 mM-$LaCl_3$ (added at 10 min, large arrow), whose influence was also monitored (□).

concerned (Fig. 6). Attention should be drawn to a similarly transient time course for the vasopressin-stimulated degradation of phosphatidylinositol 4,5-bisphosphate in hepatocytes (Kirk *et al.*, 1981); unfortunately it is not yet known whether this phosphatidylinositol 4,5-bisphosphate response is also obliterated in a subsequent hormonal challenge.

Fig. 7 illustrates the effect of temperature on phosphorylase activation, $^{45}Ca^{2+}$ fluxes, desensitization and resensitization. These data show that at 5–10°C vasopressin induces a complete desensitization, but the other effects are still not obtained, clearly indicating a complete dissociation of the cell desensitization from the glycogenolytic effect and

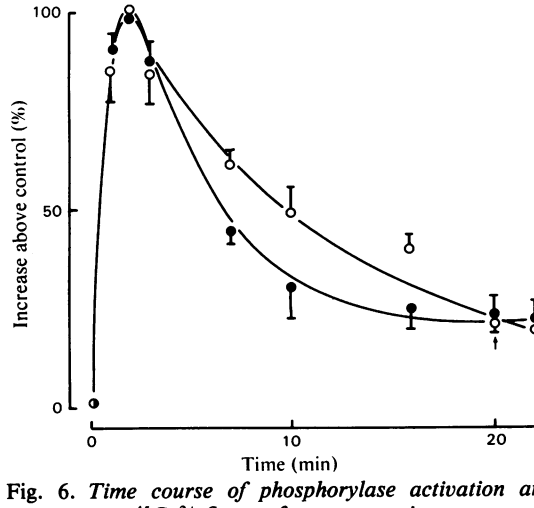


Fig. 6. *Time course of phosphorylase activation and $^{45}Ca^{2+}$ fluxes after vasopressin*

Hepatocytes were challenged with 25 nM-vasopressin. At different times thereafter, samples were taken for the assay of phosphorylase (○) and for the

measurement of the uptake of $^{45}Ca^{2+}$ added together with vasopressin (●). The arrow indicates a second addition of 25 nM-vasopressin. Values are given as means ± S.D. (where scale permits) for three experiments.

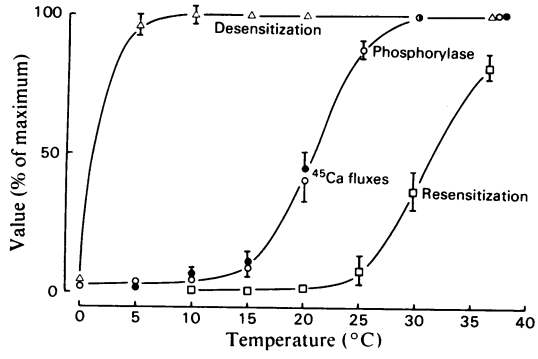
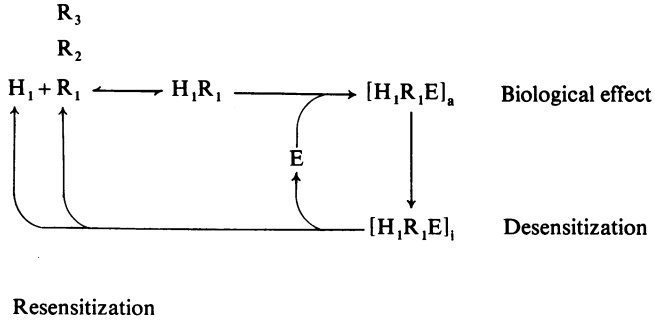


Fig. 7. *Effect of temperature on the degree of desensitization and resensitization, $^{45}Ca^{2+}$ fluxes and phosphorylase activation by vasopressin*

Hepatocytes were incubated in a Hepes-buffered saline solution, pH 7.4 (119.4 mM-NaCl, 4.8 mM-KCl, 2.6 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 10 mM-glucose and 20 mM-Hepes) at different temperatures in open glass 20 ml vials and continuously shaken. Glass vials were used instead of plastic ones to facilitate temperature equiliberation. Phosphorylase *a* levels (○) were determined in samples taken 2 min after the addition of 25 nM-vasopressin and are expressed as percentage values, taking the increase at 37°C as 100%. At 15°C and 20°C, phosphorylase *a* can attain higher levels than those obtained after 2 min, but they never exceeded 25% (15°C) or 70% (20°C). Ca^{2+} fluxes (●) were recorded as described (see under 'Methods') and are similarly expressed. The temperature-dependency of the desensitization (△) was estimated as follows. The cells were preincubated at the indicated temperatures with 25 nM-vasopressin; 20 min later, they were heated rapidly to 37°C in the presence of a second dose of vasopressin together with 2.5 mM-EGTA, and several cell samples were taken during the next 2 min for the assay of phosphorylase *a*. Calcium chelation and the use of the Hepes buffer prevented spurious activations of phosphorylase during the warming-up period; in control cells it never exceeded 10% of the maximal value obtained with vasopressin. As shown by Blackmore *et al.* (1978, 1982) and confirmed by us, this addition of EGTA does not interfere with the short-term effect of vasopressin. The degree of desensitization is presented in percentage values, as compared with control cells. To determine the temperature-dependency of the reversibility of the desensitization, the cells were incubated at 37°C in the presence of 25 mM-vasopressin; the Hepes solution used did not contain sulphate and phosphate (see under 'Methods'). Then 15 min later, 1 mM-$LaCl_3$ was added and the cells were rapidly warmed to the indicated temperature and further incubated. At 10 min thereafter they were again warmed to 37°C in the presence of 20 nM-angiotensin and 2.5 mM-EGTA. Several samples were taken during the next 2 min for estimation of the phosphorylase *a* levels; these were compared with those obtained with control cells, which had been treated in the same way but without vasopressin. The degree of resensitization (□) is expressed as percentage values, as compared with phosphorylase *a* levels of the control cells. Values are given as means ± S.D. (where scale permits) for three experiments.



Scheme 1. *Possible sequence of events leading to activation, desensitization and resensitization*

H_1 represents a hormone, R_1, R_2 and R_3 represent specific receptors and E represents the hypothetical common effector with which each hormone–receptor complex (H_1R_2, H_2R_2, H_3R_3...) has to form an active hormone–receptor–effector complex $[H_1R_1E]_a$ in order to produce a biological effect. The conversion of this active complex $[H_1R_1E]_a$ into the inactive $[H_1R_1E]_i$ form is responsible for the desensitization, and, by trapping E in $[H_1R_1E]_i$, readily accounts for its heterologous nature. Washing away the hormone (Fig. 1), displacement from its receptor by an antagonist (Figs. 4 and 5) or spontaneous degradation results in the restoration of the responsiveness, i.e. resensitization.

the Ca^{2+} movements. The desensitization observed at these low temperatures is also heterologous, since it is also directed against phenylephrine or angiotensin (results not shown). It is also noteworthy that the resensitization only occurs from about 25°C upwards, clearly indicating radically different temperature-dependencies for desensitization and resensitization.

Taken together, the data suggest a primary agonist-induced event leading to activation, followed by desensitization of the cells, which takes place independently of Ca^{2+} gating and phosphorylase activation; on removal of the agonist, resensitization can take place. We propose that this early event occurs in the plasma membrane and that it could involve the transition of an active hormone–receptor–effector complex (responsible for activation of the cell) to an inactive complex (leading to desensitization). This is tentatively illustrated by Scheme 1.

The fact that at low temperature there is a disconnection between desensitization and metabolic effects (Fig. 7) might be due either to the inability of $[HRE]_a$ to provoke sufficient Ca^{2+} movements at low temperature, or to the slowing down of the phosphorylase kinase reaction, or most probably to a combination of both.

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