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Heterologous desensitization of the cyclic AMP-independent glycogenolytic response in rat liver cells

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Vasopressin and α-adrenergic agonists are known to be potent cyclic AMP-independent Ca^{2+}-dependent activators of liver glycogen phosphorylase. When hepatocytes are pre-incubated with increasing concentrations of vasopressin or of the α-agonist phenylephrine, they become progressively unresponsive to a second addition of the respective agonist. The relative abilities of six vasopressin analogues and of five α-agonists to activate glycogen phosphorylase and to cause subsequent desensitization are highly correlated, indicating that the same vasopressin and α-adrenergic receptors are involved in both responses. About 5-times-higher peptide concentrations are needed to desensitize the cells than to activate their glycogen phosphorylase, whereas the concentrations of α-agonists required for the desensitization are only twice those needed for the activation of phosphorylase. The desensitization is not mediated by a perturbation in the agonist–receptor interaction. It is clearly heterologous, i.e. it is not agonist-specific, and must therefore involve a mechanism common to both series of agonists. The evidence for a role of Ca^{2+} movements or phosphatidylinositol turnover is briefly discussed.

A common feature to many, if not all, adenylate cyclase-coupled hormones is the transient nature of their response due to a refractoriness induced in the target cells. This desensitization is most frequently effector-specific (or homologous), although several examples of non-specific (or heterologous) desensitization have been described. Homologous desensitization suggests an alteration at the level of the relevant receptor, whereas the heterologous phenomenon seems to implicate either a decreased production or an enhanced hydrolysis of cyclic AMP, although other mechanisms should also be considered [see Lefkowitz *et al.* (1980) for a recent review]. Apart from the well established cyclic AMP-dependent regulation of liver glycogenolysis, there also is a cyclic AMP-independent glycogenolysis initiated by α-adrenergic agonists, vasopressin and angiotensin; according to the current evidence (see De Wulf *et al.*, 1980; Kirk *et al.*, 1980), the mechanism involves an increase in cytosolic $[Ca^{2+}]$, which will stimulate phosphorylase kinase and so lead to the activation of glycogen phosphorylase, the rate-limiting enzyme in glycogen breakdown. It was therefore of interest to see whether these cyclic AMP-independent glycogenolytic hormones are able to induce a desensitization of liver cells, and, if this is the case, to determine whether the desensitization is homologous or heterologous. The data show that vasopressin and α-adrenergic agonists induce a dose-dependent desensitization of the phosphorylase response in isolated rat hepatocytes. They also indicate that the same α-adrenergic or vasopressin receptors are involved in both the activation of glycogen phosphorylase and in the subsequent tachyphylaxis. Finally, it is shown that the desensitization is heterologous. Some results have been briefly described previously (Bréant *et al.*, 1981; Keppens *et al.*, 1981).

Experimental

Methods

We have used male Wistar strain albino rats (200–250g body wt.), which were allowed free access to food. Liver cells were prepared, incubated and sampled as described previously (Vandenheede *et al.*, 1976). Succinctly, the cells (approx. 5×10^6 cells/ml) were suspended in Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) gassed with O_2/CO_2 (19:1, v/v) and pre-incubated for 20min with 10mM-glucose and 1mM-bacitracin at 37°C in closed plastic 20-ml vials with continuous shaking (120 oscillations/min) in the presence of the O_2/CO_2

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mixture. The incubations with the α-adrenergic agents were done in the presence of stabilizing agents (3 mM-catechol and 1 mM-ascorbate) and of the β-antagonist propranolol (0.1 mM). The glycogenolytic potency of an agonist was estimated by the increase in phosphorylase *a* activity of the cells 90 s after the addition of increasing concentrations. To establish the desensitizing capacity, the cells were first incubated with increasing concentrations of either a vasopressin analogue or an α-agonist; 20 min later, the cells were challenged with either 1 nM-[Arg^8]vasopressin or 10 μM-phenylephrine. At that time and 90 s later, phosphorylase *a* activity of the cells was determined. The desensitizing potency was estimated from the decreasing efficiency of vasopressin or phenylephrine respectively to reactivate the enzyme.

Phosphorylase *a* activities were measured as described previously (Vandenheede *et al.*, 1976). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Chemicals

[Arg^8]Vasopressin (grade VI), adrenaline, noradrenaline, deoxyadrenaline, dopamine (3,4-dihydroxyphenethylamine), phenylephrine, propranolol and catechol were from Sigma Chemical Co, St. Louis, MO, U.S.A. [Orn^8]Vasopressin and ocytocin were from Sandoz A.G., Basel, Switzerland. [1-deamino-D-Arg^8]Vasopressin and [(*O*-ethyl-Tyr^2-Lys^8]vasopressin were kindly provided by Dr. H. Vilhardt (Ferring Pharmaceuticals, Malmö, Sweden). Mesotocin was a gift from Professor E. Kühn (Department of Zoology, University of Louvain, Belgium). Glucagon was purchased from Novo-laboratories, Copenhagen, Denmark, and angiotensin from Schwarz–Mann, Orangeburg, NY, U.S.A. Other sources have been listed previously (Vandenheede *et al.*, 1976).

Results

Desensitization by vasopressin

The possibility of a refractoriness of liver cells to the glycogenolytic action of vasopressin stems from the transient nature of the activation of glycogen phosphorylase with continued exposure to the hormone (Fig. 1); previous experiments have shown that vasopressin is fairly stable during such incubations (Cantau *et al.*, 1980). After 20–30 min, when the enzyme activity has declined to near-basal levels, re-addition of a biologically supramaximal dose of vasopressin no longer elicits a second wave of activation (Fig. 1); control cells still react to a more delayed addition of the peptide (Fig. 1), indicating that desensitization is caused by the hormone and not by mere incubation of the cells. We

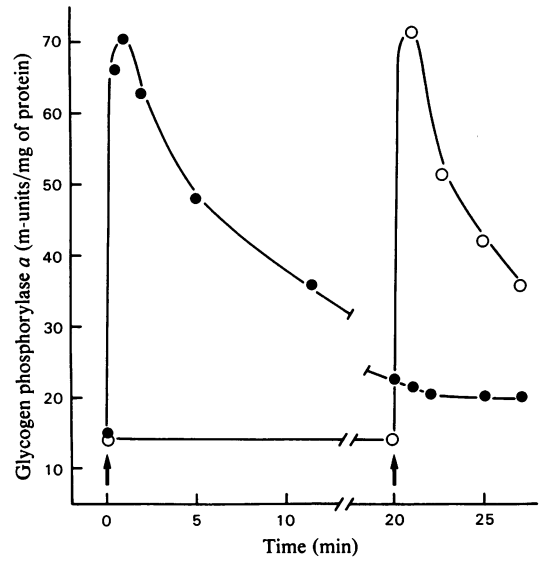

Fig. 1. *Transient nature and subsequent desensitization of the glycogenolytic response to vasopressin*
Hepatocytes were challenged with 25 nM-vasopressin at time 0 min and with 1 nM-vasopressin at time 20 min (●), or only at time 20 min with 1.5 nM-vasopressin (○). The same result was obtained when 10 nM- or 100 nM-vasopressin was added at time 20 min.

have checked that no antagonist is produced during the incubation of hepatocytes with vasopressin. Indeed, after 20–30 min, the supernatant of the incubation still contains an unmodified vasopressin activity when tested with a fresh cell suspension (results not shown).

Previous experiments have shown that the activation of glycogen phosphorylase by [Arg^8]vasopressin is dose-dependent with a K_a (concentration for half-maximal activation) of approx. 0.2 nM (Keppens & De Wulf, 1979; Cantau *et al.*, 1980). We established the dose-dependency of the desensitization in the following way. Hepatocytes were pre-incubated with increasing concentrations of vasopressin for 20 min and were then challenged with an otherwise (i.e. with control cells) maximally effective dose of vasopressin; the decrease in the (expected) phosphorylase response was taken as a measure of the extent of desensitization. Such an experiment is shown in Fig. 2. Half-maximal inhibition is obtained at a concentration (K_i) of about 1.5 nM-[Arg^8]vasopressin.

To establish whether the desensitization is mediated by the same receptors as those shown to be involved in the glycogenolytic response (Keppens & De Wulf, 1979; Cantau *et al.*, 1980), we have used

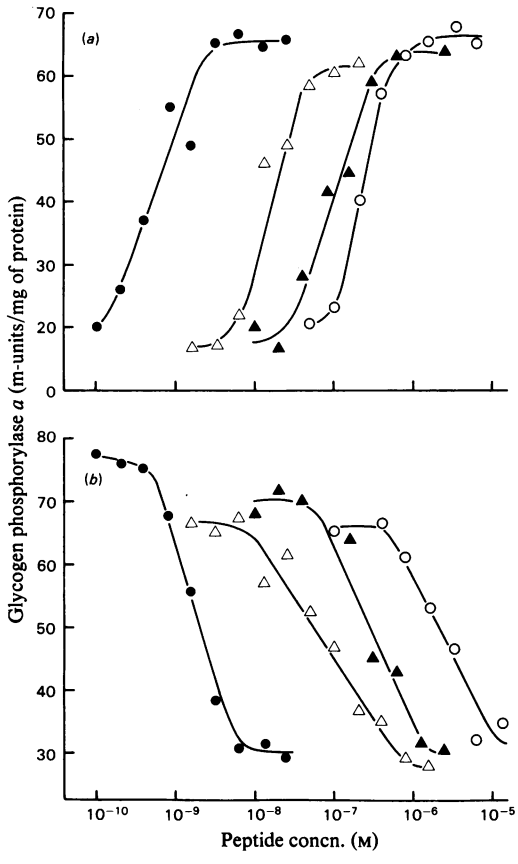

Fig. 2. *Dose-dependency of several vasopressin analogues for phosphorylase activation and subsequent desensitization*

Hepatocytes were incubated with increasing concentration of several vasopressin analogues. (*a*) The glycogenolytic potency was estimated 90s later. (*b*) The desensitizing potency was estimated 20min later by measuring the phosphorylase *a* activities 90s after the addition of 1nM-[Arg^8]vasopressin. Symbols: ●, [Arg^8]vasopressin; △, [Tyr^2-*O*-ethyl-Lys^8]vasopressin; ▲, mesotocin; ○, [1-deamino-D-Arg^8]vasopressin.

vasopressin analogues to compare their glycogenolytic (estimated by their K_a) and desensitizing (estimated by their K_i) capacities. Fig. 2 illustrates an experiment with four of these analogues. It is clear that the more active they are in activating glycogen phosphorylase, the more potent they are in desensitizing against [Arg^8]vasopressin. The mean values for the K_a and K_i are listed in Table 1 and the strong correlation ($r = 0.998$, $P < 0.001$) is illustrated in Fig. 3. It seems therefore that the same receptors are implicated in both phenomena. On average we need a 5-fold higher concentration of peptide to desensitize the hepatocytes than to activate their glycogen phosphorylase.

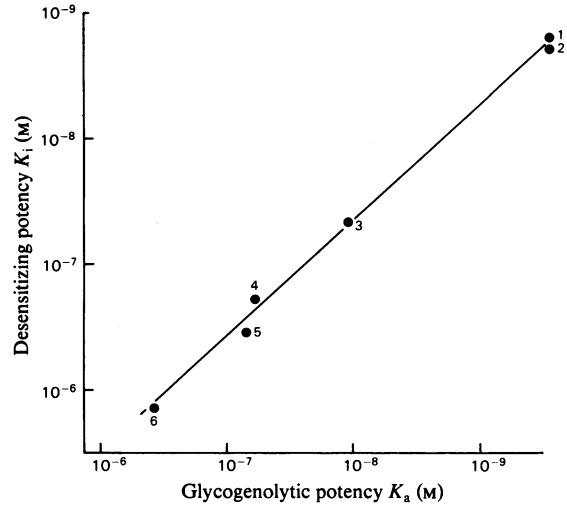

Fig. 3. *Correlation between the glycogenolytic (K_a) and desensitizing (K_i) potencies of vasopressin analogues*

The numbers identify the peptides listed in Table 1.

Densensitization by α-adrenergic agents

Similar experiments to those described for vasopressin were done with α-agonists. Fig. 4 illustrates the transient nature of the phosphorylase activation obtained with phenylephrine followed by a refractoriness of the cells to a second stimulation. The failure of Aggerbeck *et al.* (1980) to observe a desensitization with noradrenaline can be ascribed, as they also suspected, to rapid degradation of the hormone: we found it mandatory to include catechol and ascorbic acid and we used about a four-times more diluted cell suspension. To establish whether the same receptor population is involved in both phenomena, we studied the dose-dependency of five α-adrenergic agents according to the protocol described for vasopressin. Typical dose–effect curves, depicted in Fig. 5, clearly suggest the same rank-order in potency. The mean values for K_a and K_i are listed in Table 2 and graphically correlated in Fig. 6. Again the highly significant ($P < 0.001$) correlation ($r = 0.994$) points clearly to the involvement of the same α-receptors for both the activation of glycogen phosphorylase and subsequent desensitization of this enzymic response. At variance with the vasopressin family, the α-agonist concentration needed to desensitize the hepatocytes is only twice that required to activate glycogen phosphorylase.

Nature of desensitization

Several lines of evidence are in favour of the heterologous character of the desensitization by vasopressin and phenylephrine. Vasopressin desensitizes hepatocytes against itself but also, and with the same K_i, against phenylephrine and angiotensin (all three acting in a cyclic AMP-independent way)

Table 1. *Comparison of the glycogenolytic (K_a) and desensitizing (K_i) potencies of vasopressin structural analogues*
The K_a and K_i values were computed from experiments such as those illustrated in Fig. 2. Results are means ± S.E.M.

Analogue	*n*	K_a (nM)	K_i (nM)
1. [Arg^8]Vasopressin	5	0.284 ± 0.06	1.58 ± 0.48
2. [Orn^8]Vasopressin	3	0.28 ± 0.13	1.98 ± 0.9
3. [Tyr^2-*O*-ethyl-Lys^8]Vasopressin	2	11.1 ± 3.0	48.6 ± 1.9
4. Ocytocin	2	58.0 ± 11	184 ± 9.3
5. Mesotocin	3	72.1 ± 24	341 ± 160
6. [1-deamino-D-Arg^8]Vasopressin	2	366 ± 80	1500 ± 430

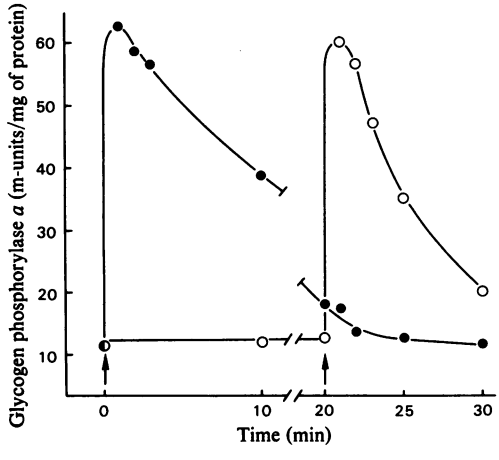

Fig. 4. *Transient nature and subsequent desensitization of the glycogenolytic response to phenylephrine*
Hepatocytes were challenged with 10 μM-phenylephrine at time 0 and 20 min (●), or only at time 20 min (○).

Table 2. *Comparison of the glycogenolytic (K_a) and desensitizing (K_i) capacities of* α*-adrenergic agonists*
The K_a and K_i values were computed from experiments such as those illustrated in Fig. 5. Results are means ± S.E.M.

Agonist	*n*	K_a (μM)	K_i (μM)
1. Adrenaline	3	0.067 ± 0.029	0.11 ± 0.05
2. Noradrenaline	4	0.287 ± 0.025	0.208 ± 0.10
3. Phenylephrine	6	1.55 ± 0.81	3.0 ± 1.05
4. Deoxyadrenaline	3	3.26 ± 1.35	8.16 ± 2.28
5. Dopamine	4	85.0 ± 20.7	208 ± 127

but not against glucagon, whose action is mediated by cyclic AMP (Fig. 7). Similar results have been obtained with phenylephrine (results not shown). The desensitization by angiotensin proved more

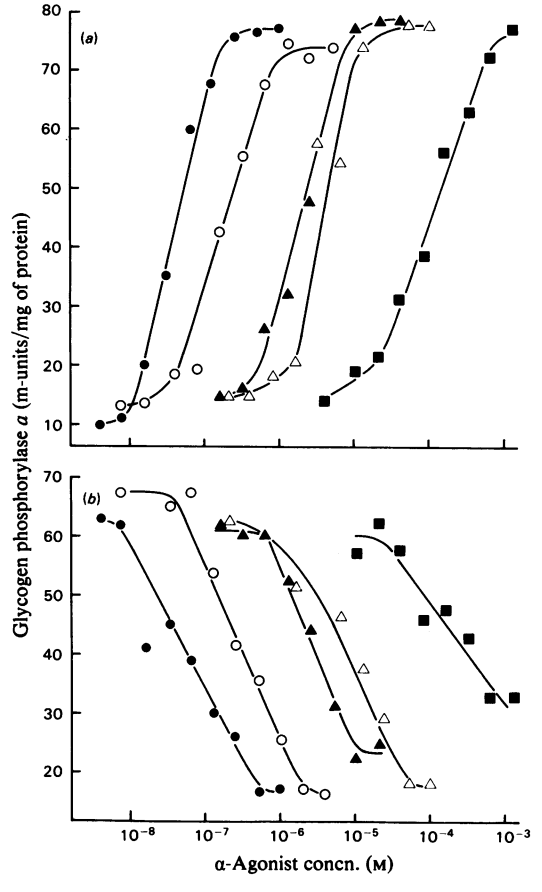

Fig. 5. *Dose-dependency of several* α*-adrenergic agonists for phosphorylase activation and subsequent desensitization*
Hepatocytes were incubated with increasing concentrations of several α-adrenergic agonists. (*a*) The glycogenolytic potency was estimated 90 s later. (*b*) The desensitizing potency was estimated 20 min later by measuring the phosphorylase *a* activities 90 s after the addition of 10 μM-phenylephrine. Symbols: ●, adrenaline; ○, noradrenaline; ▲, phenylephrine; △, deoxyadrenaline; ■, dopamine.

difficult to study because of the fairly extensive hormonolysis in the absence of 8-hydroxyquinoline sulphate, which by itself activates glycogen phosphorylase (in a yet unknown manner). Neither phenylephrine nor angiotensin interfere with [^{3}H]-vasopressin binding to hepatocytes, as expected from the receptor specificity indicated by Cantau *et al.* (1980); similarly, the [^{3}H]angiotensin binding to hepatocytes is unmodified by vasopressin or phenylephrine (results not shown). The desensitization is therefore heterologous and is situated beyond the receptor level at some point common to the three cyclic AMP-independent glycogenolytic agents.

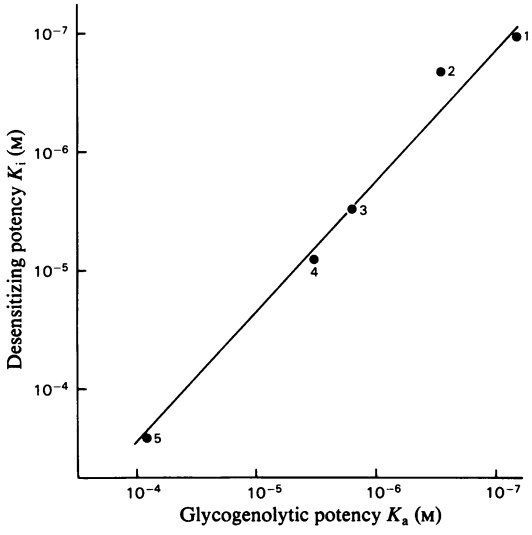

Fig. 6. *Correlation between the glycogenolytic (K_a) and desensitizing (K_i) potencies of α-adrenergic agonists*
The numbers identify the agonists listed in Table 2.

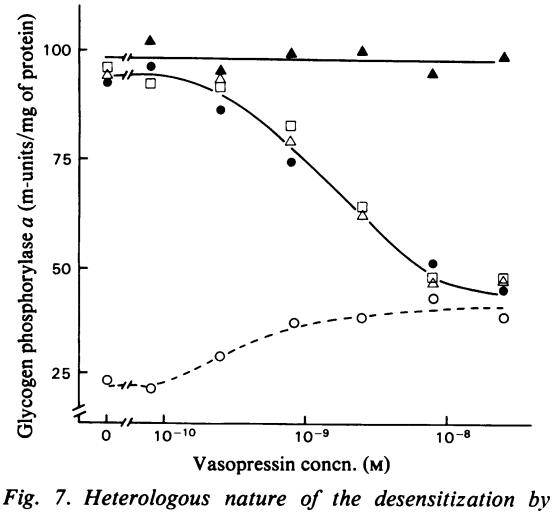

Fig. 7. Heterologous nature of the desensitization by vasopresin
Hepatocytes were incubated for 20 min in the presence of increasing concentrations of vasopressin. Phosphorylase *a* activities were then estimated (○) and also 90 s after the addition of 1 nM-vasopressin (●), 2 nM-angiotensin (△), 10 μM-phenylephrine (□) or 5 nM-glucagon (▲).

Discussion

The data provide unambiguous evidence for the desensitization of a hormonally regulated metabolic response that is not mediated by cyclic AMP. As indicated in the Introduction, the vasoactive peptides vasopressin and angiotensin and the α-adrenergic agonists lead to an activation of liver glycogen phosphorylase by a cyclic AMP-independent but Ca^{2+}-mediated mechanism. We have shown that vasopressin and α-agonists cause a transient activation of glycogen phosphorylase followed by a dose-dependent dampening of this enzymic response to a second agonist application. This decreased phosphorylase activation is not due to the production of an antagonist and is restricted to those agonists that are Ca^{2+}-dependent. Although the desensitization is heterologous (e.g. vasopressin desensitizes the hepatocytes to itself and to angiotensin and phenylephrine as well) it is not directed towards glucagon, whose action is mediated by cyclic AMP.

The use of structural analogues of vasopressin enables us to conclude that the same receptors are involved in the activation of glycogen phosphorylase and in the subsequent desensitization (Fig. 3, Table 1). According to the terminology proposed by Michell *et al.* (1979), these should be named V_1-vasopressin receptors; their interaction with the peptide controls Ca^{2+} movements, phosphorylase activation, phosphatidylinositol turnover and, as shown in the present paper, desensitization. By employing an array of α-agonists we were able to show that the same α-receptors mediate both the phosphorylase activation and the ensuing desensitization (Table 2, Fig. 6). Since α_1-receptors govern liver glycogenolysis, phosphatidylinositol turnover and presumably also Ca^{2+} movements (see Fain & Garcia-Sainz, 1980; Schmelck & Hanoune, 1980), the same α_1-receptors also seem to mediate the tachyphylaxis phenomenon.

The heterologous nature of the desensitization implies that it would be due to the 'inactivation' of a common effector (messenger) beyond the agonist–receptor interaction. According to the current evidence, this could be obtained either by depletion of Ca^{2+} (or its unavailability) in the same pool or by

lack of phosphatidylinositol in a critical compartment. Indeed, according to several authors (Blackmore *et al.*, 1978, 1979; Chen *et al.*, 1978; Babcock *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Murphy *et al.*, 1980; Poggioli *et al.*, 1980; Barritt *et al.*, 1981), hepatocytes have stores from which Ca^{2+}, required for the activation of glycogen phosphorylase, can be mobilized; a depletion or unavailability of these stores caused by a first stimulus would lead to a refractoriness to a subsequent stimulus. On the basis of the work of Michell and his co-workers (Michell, 1975; Kirk *et al.*, 1980), it could be equally possible that heterologous desensitization is caused by the degradation of phosphatidylinositol (in the plasma membrane?); as a consequence, gating of extracellular Ca^{2+} (or mobilization of Ca^{2+} from an intracellular pool) would come to a stop. A way to distinguish between these two possibilities is to compare the dose-dependency of the desensitization to that of the enhancement of phosphatidylinositol turnover and Ca^{2+} movements; the closer the desensitizing potency of an agonist to its ability to influence one of these two processes, the more likely this particular process would be involved in the desensitizing effect. The dose-dependency of vasopressin for the desensitization is close to that for the control of phosphatidylinositol turnover (Kirk *et al.*, 1979, 1981), whereas the control of calcium metabolism (Keppens & De Wulf, 1981) is obtained at 5-fold lower concentrations. Interestingly, binding of [^{3}H]-vasopressin to intact hepatocytes also occurs at concentrations (Cantau *et al.*, 1980) close to those responsible for the desensitization and the phosphatidylinositol response. This would indicate the desensitization process and the control of phosphatidylinositol turnover to be intimately connected with hormone binding and therefore possibly with the primary action on the cell membrane.

The desensitizing potency of the α-agonists appears similar to their reported ability to govern Ca^{2+} movements (El-Refai *et al.*, 1979; Murphy *et al.*, 1980; Keppens & De Wulf, 1981). Whether phosphatidylinositol turnover is implicated in the desensitization cannot yet be assessed on the basis of the data available (Tolbert *et al.*, 1980).

Whatever the mechanism of the desensitization, its elucidation will help to pinpoint the primary impact of the cyclic AMP-independent Ca^{2+}-dependent glycogenolytic agents. Further progress will depend on a more detailed analysis of phosphatidylinositol and calcium content and metabolism during the activation and desensitization stages.

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