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Time-dependent conversion of α_1- to β-adrenoceptor-mediated glycogenolysis in isolated rat liver cells: Role of membrane phospholipase A_2

(regulation of adrenoceptors/lipomodulin/melittin)

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ABSTRACT **Incubation of isolated rat liver cells in a serum-free buffer leads to the reduction of the glycogenolytic effect of phenylephrine and the simultaneous emergence of a glycogenolytic response to isoproterenol within 4 hr. This conversion of the adrenergic activation of phosphorylase from an α_1- to a β-adrenoceptor-mediated response is associated with no change in the glycogenolytic response to the calcium-linked activator vasopressin, and a reduction of the glycogenolytic response to the cAMP-linked activator glucagon. *In vitro* incubation of hepatocytes does not influence the density or affinity of [^{3}H]prazosin-labeled α_1-receptors and [^{3}H]CGP-12177-labeled β-receptors. In cells preincubated for 4 hr, a further 30-min incubation with 50 nM lipomodulin, an endogenous inhibitor of membrane phospholipase A_2 (EC 3.1.1.4), reverses the adrenergic activation of phosphorylase from a β- to an α_1-receptor-mediated event, whereas in freshly isolated cells lipomodulin does not affect the predominant α-receptor response. Conversely, exposure of freshly isolated cells to a monoclonal antibody to lipomodulin in the presence of 10 μM phenylephrine, or to melittin, an activator of phospholipase A_2, at 2 μg/ml, results in the suppression of the effect of phenylephrine and the emergence of a response to isoproterenol within 30 min. It is proposed that coupling of hepatic α_1- and β-adrenoceptors to postreceptor pathways is regulated in an inverse reciprocal manner by changes in membrane phospholipase A_2 activity.**

Catecholamines produce a multiplicity of biological effects by altering the activity of one of two biochemical pathways: a cyclic AMP (cAMP)-dependent cascade mechanism, or a cAMP-independent pathway triggered by changes in intracellular calcium. Activation of adenylate cyclase is mediated by β-adrenoceptors, whereas activation of the calcium-dependent pathway occurs through α_1-adrenoceptors. In some tissues these two pathways converge on a common end point to induce the same biological response, such as increased myocardial contractility or hepatic glycogenolysis. In certain physiological conditions there is a shift in the relative contribution of α_1- and β-receptors to the net response to catecholamines (1–7). The rat liver has become an attractive test system for studying the mechanism of this apparent interconversion of α_1 and β-adrenergic responses. In the adult male rat the glycogenolytic response to catecholamines is mediated by α_1-receptors (8), but it is converted into a β-receptor-mediated event in certain conditions, such as hypothyroidism (9–14), glucocorticoid deficiency (15), partial hepatectomy (16), cholestasis (16), treatment with carcinogens (17), young age (18), and primary culturing of hepatocytes (19). The latter is a particularly attractive system for further studies, because the change in receptor response develops within a few hours and occurs under *in vitro* conditions. In all these experimental models, the condition that promotes increased β-receptor dominance is associated with a lower level of cellular differentiation. This may suggest that conversion of the α_1- to β-adrenergic response is mediated through the induction of a final common pathway by the various stimuli.

One of the factors implicated in changes in the adrenergic response pattern of rat liver is glucocorticoids (15). Recently, it has become evident that most of the biological effects of glucocorticoids, including their effects on cell differentiation, are mediated indirectly, by the induction of the synthesis of an endogenous protein inhibitor of membrane phospholipase A_2 (EC 3.1.1.4) (20–22). Two such proteins have been identified: lipomodulin, a 40,000 molecular weight protein purified to near homogeneity (23), and macrocortin (21), which is probably an active fragment of lipomodulin (24). By using purified lipomodulin and a monoclonal antibody against lipomodulin, we provide evidence that changes in membrane phospholipase A_2 activity may mediate the apparent interconversion of hepatic α_1- and β-adrenergic responses that occurs during *in vitro* incubation of rat liver cells. The change in receptor response appears to involve inverse changes in the coupling of α_1- and β-receptors to postreceptor pathways.

METHODS AND MATERIALS

Hepatocytes. Isolated hepatocytes of adult male Sprague-Dawley rats (300–350 g) were prepared by a collagenase perfusion technique (11, 13). The cells were suspended in Krebs–Henseleit buffer containing 40 mM glucose and 1.5% Difco gelatin, and were incubated at 37°C under an atmosphere of 95% O_2/5% CO_2, with continuous shaking at 100 cycles per min. After a 30-min preincubation period necessary to achieve stable basal levels of phosphorylase activity (11), the cell suspension was divided into two batches. Aliquots from the first batch were used immediately (0-hr cells) to determine drug and hormone effects on glycogen phosphorylase *a* activity or to prepare crude or purified membranes for radioligand binding (see below). The remaining cells were incubated for a further 4 hr at 2 mg packed wet weight of cells per ml of buffer, under conditions described above. At the end of this period, aliquots of this cell suspension (4-hr cells) were used the same way as 0-hr cells.

Determination of Phosphorylase *a* Activity. Aliquots of liver cell suspension (2 mg wet weight per ml) were incubated in stoppered Erlenmeyer flasks for 30 min at 37°C with continuous shaking and under an atmosphere of 5% CO_2 in O_2. During this period the cells received vehicle only, or substances that influence membrane phospholipase A_2 activity, as indicated. Ten-milliliter aliquots in each treatment group then received vehicle or three to four different concentrations of *l*-isoproterenol or *l*-phenylephrine. Three minutes

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later, when phosphorylase *a* activity is at plateau (11), the aliquots were centrifuged (30 sec), the supernatants were aspirated, and the cell pellets were immediately homogenized in ice-cold phosphorylase assay buffer (11, 13). The homogenate was centrifuged at 3000 × *g* for 10 min, and phosphorylase *a* activity was assayed in duplicate aliquots of the supernatant by measuring the incorporation of [^{14}C]glucose 1-phosphate into glycogen (11, 13). Units of enzyme activity are nmol of [^{14}C]glucose incorporated into glycogen per min per mg of protein. Protein in the 3000 × *g* supernatant was measured by the method of Lowry (25).

Ligand Binding Assays. Adrenoceptor binding sites were quantified in crude homogenates as well as in purified plasma membranes obtained from 0-hr and 4-hr cells. Cells were centrifuged at 50 × *g* for 2 min, resuspended in 20 vol of ice-cold homogenizing solution containing 1 mM $NaHCO_3$ and 1 mM EGTA, and homogenized in a Potter–Elvehjem homogenizer by eight strokes of a Teflon pestle. The homogenate was centrifuged twice at 5000 × *g* for 10 min, and the final pellet was resuspended in buffer containing 50 mM Tris·HCl and 4 mM $MgSO_4$ for binding assays in crude homogenates. For preparation of purified plasma membranes, the crude homogenate was mixed with 2 vol of 69% (wt/vol) sucrose, overlayed with 42.3% sucrose, and centrifuged at 100,000 × g_{max} for 2 hr at 4°C. The plasma membranes floating on top were resuspended in 50 mM Tris·HCl/4 mM $MgSO_4$. The pH of this buffer was adjusted to 7.4 for α_1-receptor binding assays or to 8.0 for β-receptor binding assays. [^{3}H]Prazosin (13) and [^{3}H]CGP-12177 (26) were used to label hepatic α_1- and β-receptors, respectively. Binding was assayed in duplicate aliquots containing 0.1 mg (α_1) or 1.0 mg (β) of membrane protein in 1.0 ml. Incubations were for 30 min at 31°C and were terminated by vacuum filtration onto Whatman GF/C filters, followed by three 4-ml washes with 10 mM Tris·HCl buffer. Specific binding was defined as the difference between binding in the presence and absence of 400 μM *l*-norepinephrine for α_1- or 300 μM *l*-isoproterenol for β-receptors. Binding isotherms composed of five to eight points were analyzed by Scatchard plots.

Preparation of Lipomodulin and Monoclonal Antibody to Lipomodulin. Lipomodulin was purified from culture medium in which rabbit peritoneal neutrophils had been incubated in the presence of 1 μM fluocinolone acetonide for 16 hr (20), as described earlier (23). A monoclonal anti-lipomodulin antibody was identified among antibodies raised against rat cerebral synaptosomes (27). The antibody prevented the *in vitro* inhibition of phospholipase A_2 by lipomodulin. One milliliter of the undiluted, antibody-containing ascites fluid bound 1.5 mg of lipomodulin.

Materials. [^{3}H]Prazosin (18.8 Ci/mmol; 1 Ci = 37 GBq) and α-D-[U-^{14}C]glucose 1-phosphate (200 mCi/mmol) were from New England Nuclear, [^{3}H]CGP-12177 (42.6 Ci/mmol, [4-(*tert*-butylamino-2-hydroxypropoxy)benzimidazole -2-on·HCl)] was from Amersham. Glucagon, vasopressin (grade VIII synthetic arginine-vasopressin), melittin, *l*-norepinephrine, *l*-phenylephrine, and *l*-isoproterenol were from Sigma. Other chemicals were from usual commercial sources.

RESULTS

In agreement with earlier observations (8, 13), in freshly isolated rat liver cells the adrenergic activation of phosphorylase is mediated by an α_1-receptor: the α_1-receptor agonist phenylephrine is a potent stimulator with a pD_2 ($-\log EC_{50}$) of 6.58 ± 0.21, whereas the β-adrenergic agonist isoproterenol is ineffective (Fig. 1 *Upper*). After incubation of cells for 4 hr, there is a striking change in the response pattern from α_1 to β: phenylephrine loses both potency (pD_2 = 5.64 ± 0.30, $P < 0.005$) and efficacy, and isoproterenol becomes a

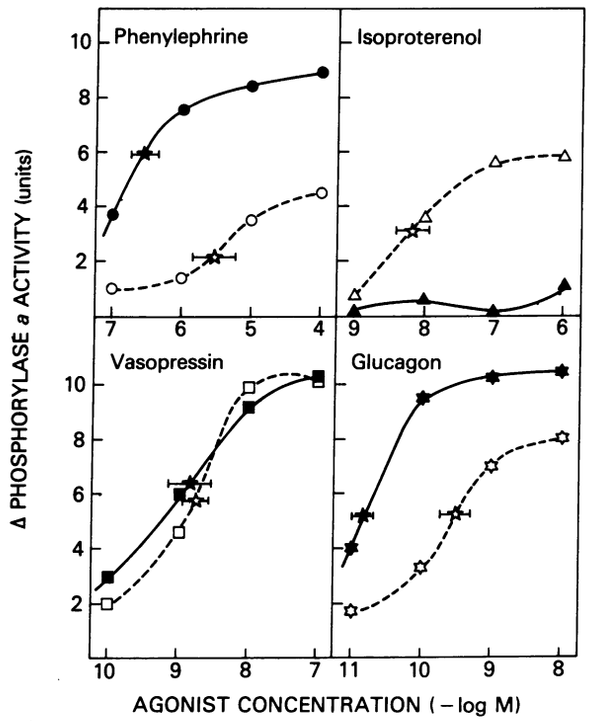

FIG. 1. Effects of *in vitro* incubation of isolated rat liver cells on phosphorylase activation by glycogenolytic hormones. Cells were isolated and incubated, and the activity of phosphorylase *a* was determined. Solid lines and filled symbols, 0-hr cells; broken lines and open symbols, 4-hr cells. Points represent means of eight (isoproterenol and phenylephrine) or four experiments (glucagon, vasopressin). Five-pointed stars and horizontal bars indicate mean EC_{50} ± SEM. Basal phosphorylase *a* activity was 12.3 ± 1.1 and 11.3 ± 0.9 units for 0-hr and 4-hr cells, respectively.

potent activator (pD_2 = 8.10 ± 0.22). Time course studies indicate that the above changes become significant after 2 hr and reach their maximum at 4 hr. To test the selectivity of this time-dependent change in adrenoceptor response, phosphorylase activation by vasopressin and glucagon were also measured in 0-hr and 4-hr cells. Vasopressin acts through a cAMP-independent mechanism similar to that involved in the α_1-adrenergic response (28), whereas glucagon activates phosphorylase through the same cAMP-dependent cascade as isoproterenol. As illustrated in Fig. 1 *Lower*, incubation of cells for 4 hr did not influence their response to vasopressin, whereas the potency of glucagon was markedly reduced (pD_2 = 10.82 ± 0.15 for 0-hr and 9.57 ± 0.25 for 4-hr cells, $P < 0.001$), in sharp contrast to the simultaneous emergence of the β-adrenergic response to isoproterenol. Thus, the effects of glycogenolytic hormones acting via the same second messenger systems as α_1- and β-adrenergic agonists are not similarly affected by prolonged incubation of hepatocytes.

Hepatic α_1- and β-receptors were also identified directly by ligand binding. In membrane preparations of rat liver, [^{3}H]prazosin labels a single class of noninteracting binding sites with the specificity of α_1-adrenoceptors (13, 29). In preparations obtained from 0-hr and 4-hr cells, there was no statistically significant difference in either the density or the affinity of [^{3}H]prazosin-labeled α_1-receptors (Table 1). Similarly, incubation of cells for 4 hr did not influence the B_{max} or K_d of [^{3}H]CGP-12177-labeled β-receptors in crude membranes or the binding of 2 nM radioligand in purified membranes, where full binding isotherms were not determined due to the limited amounts of membrane protein (Table 1). This recently developed hydrophilic radioligand (26) was used because its binding to liver membranes shows the ex-

Table 1. Effects of prolonged *in vitro* incubation of rat liver cells on the density and affinity of α_1- and β-receptor binding sites

	Crude homogenate		Plasma membrane	
Cells	B_{max}, fmol/mg	K_d, pM	B_{max}, fmol/mg	K_d, pM
		α_1-Adrenoceptors		
0-hr	84.1 ± 17.1	104 ± 22	330.4 ± 96.0	83 ± 24
4-hr	65.4 ± 11.0	99 ± 28	325.8 ± 87.2	102 ± 27
		β-Adrenoceptors		
0-hr	8.55 ± 0.98	700 ± 100	11.20 ± 1.65*	
4-hr	7.96 ± 1.42	800 ± 300	10.90 ± 1.90*	

α_1-Receptors were assayed with [^{3}H]prazosin and β-receptors with [^{3}H]CGP-12177. Specific binding of [^{3}H]prazosin (0.05–1.0 nM) was 90–75% in crude homogenates and 95–85% in purified membranes. Specific binding of [^{3}H]CGP-12177 (0.25–4 nM) was 75–50% in homogenates and 72% at 2 nM in plasma membranes. In each liver, binding was tested in both 0-hr and 4-hr cells. Values are given ± SEM. By the paired *t* test, none of the B_{max} of K_d values were significantly different between the two treatment groups. The total number of livers tested for each receptor was six with crude homogenate and five with plasma membrane.

*Due to the small amount of purified membrane (6–8 mg per treatment group per liver), β-receptor binding could be assayed at only a single concentration, 2 nM.

pected stereoselectivity for both agonists and antagonists (data not shown). In earlier attempts to label hepatic β-receptors with lipid-soluble radioligands such as [^{3}H]dihydroalprenolol or ^{125}I-labeled hydroxybenzylpindolol, we were unable to show stereoselectivity for agonists, although stereoselectivity for antagonists was readily identified. Similar problems were reported by others (30). The above results indicate that the selective inverse changes in α_1- and β-receptor responses are not due to altered density or affinity of the respective binding sites, although more subtle changes in receptors have not been excluded.

Inverse changes in α_1- and β-adrenergic responses, similar to those seen in 4-hr cells, have been observed in adrenalectomized rats, and the effects of adrenalectomy were reversed by *in vivo* treatment with a glucocorticoid (15). Because the mechanism of the altered response may be similar in these two experimental models, we tested the *in vitro* effects of lipomodulin on the adrenoceptor response pattern of isolated liver cells. The results in Fig. 2 illustrate that incubation of 4-hr cells for an additional 30 min with 50 nM lipomodulin nearly completely suppresses the effect of isoproterenol and increases the potency of phenylephrine to that observed in 0-hr cells. Preabsorption of lipomodulin with an excess of lipomodulin antibody eliminated the above effects, indicating their specificity. When lipomodulin was incubated with 0-hr cells, the effect of phenylephrine was not significantly altered and isoproterenol remained ineffective (not shown). These findings suggest that the time-dependent conversion of the adrenoceptor response is due to a parallel increase in membrane phospholipase A_2 activity, possibly caused by the loss of an endogenous phospholipase inhibitory substance, such a lipomodulin. Therefore, we tested whether the change in receptor response can be acutely induced by treating freshly isolated liver cells with an antibody to lipomodulin.

When 0-hr cells were incubated for 10 min with a 1:50 dilution of a monoclonal antibody to lipomodulin and then washed for 30 min, there was only a minor reduction in the potency of phenylephrine, and isoproterenol remained ineffective. However, when the cells were first exposed to 10 μM phenylephrine for 10 min and then monoclonal antibody was added for an additional 10 min, the effect of phenylephrine tested after the washout period was markedly reduced, and isoproterenol emerged as a potent agonist (Fig. 3).

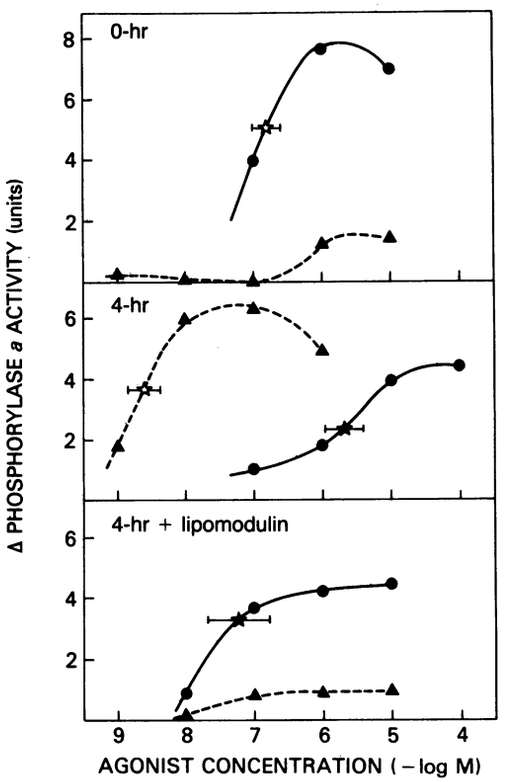

FIG. 2. Reversal by lipomodulin of the time-dependent conversion of the hepatic α_1- to a β-adrenergic response. The effects of phenylephrine (filled circles, solid line) and isoproterenol (triangles, broken line) were tested in freshly isolated cells (*Top*), in cells incubated for 4 hr (*Middle*), and in 4-hr cells incubated for a further 30 min with 50 nM purified lipomodulin (*Bottom*). Means of four experiments are shown. Stars and horizontal bars indicate mean EC_{50} ± SEM. Baseline phosphorylase *a* activity was 13.4 ± 1.3 units (0-hr cells), 11.2 ± 1.1 units (4-hr cells), and 14.4 ± 1.8 units (4-hr cells treated with lipomodulin).

Similar effects were observed when 0-hr cells were incubated for 30 min with melittin, a polypeptide activator of phospholipase A_2 (ref. 31, Fig. 4) at 2 μg/ml. Melittin also caused a substantial increase in basal phosphorylase *a* activity, which may be related to its action as a calcium ionophore. However, this latter action is unlikely to account for the observed shift from α_1- to β-receptor activity, because (*i*) it does not explain the emerging response to isoproterenol, and (*ii*) the similar effects of lipomodulin antibody are associated with a decrease rather than an increase in basal phosphorylase *a* activity (see Fig. 3).

DISCUSSION

Previous work has shown that primary culturing of rat liver cells results in a rapid conversion of the adrenergic activation of glycogenolysis from an α_1- and β-receptor response (19). The present results confirm and extend these findings and provide evidence to suggest that changes in membrane phospholipase A_2 activity mediate the apparent interconversion of the adrenoceptor response. The endogenous phospholipase A_2 inhibitor, lipomodulin, reverses the time-dependent shift in adrenoceptor response. Conversely, a monoclonal antibody to lipomodulin, or melittin, an activator of phospholipase A_2, acutely induces such a shift in freshly isolated cells. A possible explanation of these findings is that primary culturing or, as in the present study, simple incubation of hepatocytes in a buffer leads to a gradual loss of an endogenous inhibitor of membrane phospholipase A_2 activity. Another possibility is a time-dependent increase

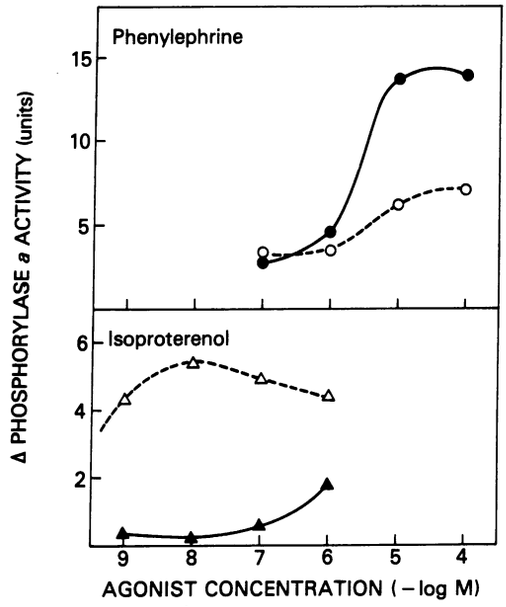

FIG. 3. Effects of preincubation of cells with lipomodulin antibody in the presence of phenylephrine on the adrenergic activation of phosphorylase *a*. Freshly isolated rat liver cells were incubated with a 1:50 dilution of a monoclonal antibody to lipomodulin in the presence of 10 μM phenylephrine and then washed for 30 min before the effects of phenylephrine and isoproterenol were tested. Filled symbols and solid lines, control cells; open symbols and broken lines, antibody-treated cells. Means of two experiments are shown. Basal phosphorylase activity was 12.2 and 9.4 units in control and antibody + phenylephrine-treated cells, respectively.

in the synthesis of an endogenous phospholipase activator, which may be similar in action to a recently reported glucosteroid-response-modifying factor (32). This latter mechanism may account for the reported inhibition of the time-dependent conversion of hepatic α- to β-receptor responses by protein synthesis inhibitors (19, 33). One or both of these mechanisms may be operating in the liver cell, and the resulting increase in membrane phospholipase A_2 activity appears to mediate the conversion of the α_1- to a β-receptor type response. The presence of a lipomodulin-like substance in freshly isolated liver cells has not been directly demonstrated, but its existence is suggested by the effects of the lipomodulin antibody. The observation that the antibody-induced shift from α_1- to β-adrenergic reactivity occurs only when α-receptors are simultaneously activated suggests a mutual interaction between membrane phospholipase A_2 and adrenoceptors. Thus, prolonged stimulation of α_1-receptors would facilitate the increase in enzyme activity that, in turn, suppresses the receptor response. Interestingly, prolonged incubation of rat liver cells with isoproterenol was reported to prevent the time-dependent shift from α_1- to β-receptors (19, 34). This could mean that α_1- and β-receptors have opposite regulatory influences on membrane phospholipase A_2 activity.

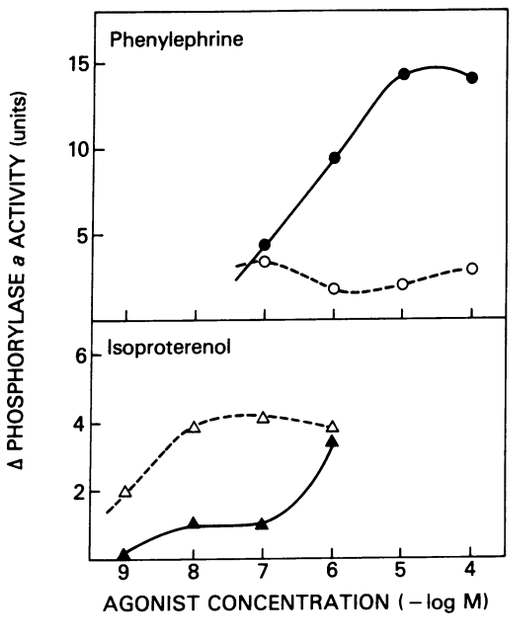

FIG. 4. Effects of Melittin on the adrenoceptor-mediated activation of phosphorylase *a* in freshly isolated rat liver cells. Freshly isolated hepatocytes were incubated for 30 min with vehicle only (filled symbols, solid line) or with melittin at 2 μg/ml (open symbols, broken line). Means of three experiments are shown. Baseline phosphorylase *a* activity was increased by melittin from 10.6 ± 2.3 to 18.6 ± 2.1 units.

Unlike isoproterenol, glucagon is a potent glycogenolytic agent in freshly isolated hepatocytes, and its effect is not increased but rather decreased with time (Fig. 1). This indicates that the adenylate cyclase–cAMP system is not deficient in rat liver cells, and the mechanism of the emergence of a β-adrenergic response after 4 hr is in place before the generation of the second messenger signal. Similarly, the unchanged effect of the calcium-linked activator, vasopressin, indicates that cellular pools of releasable calcium or the Ca^{2+}-sensitive cascade activated by it are unaffected by prolonged incubation of the cells. Thus, the time-dependent decrease in the α_1-receptor response also must occur at a site before the release of the second messenger calcium. This leaves the receptors or their transduction mechanisms as possible targets for regulation. The very rapid onset of the inverse changes in receptor response, particularly after incubation of cells with substances that modify membrane phospholipase A_2 activity (<30 min), makes it unlikely that these changes are due to altered synthesis or turnover of receptors. This is further discounted by the finding that neither the density nor the affinity of α_1- and β-receptor binding sites was influenced by 4 hr of incubation of the cells (Table 1). Therefore, the most likely targets for the regulatory influence of membrane phospholipase A_2 are the transduction mechanisms for α_1- and β-receptors. The reported uncoupling of cardiac β-receptors from adenylate cyclase by melittin without a change in the binding sites (35) is in agreement with such a possibility. Although this effect is opposite in direction to the increase in β-adrenergic reactivity by melittin in the liver, conditions such as hypothyroidism are also known to influence cardiac and hepatic adrenoceptors in opposite ways (4, 13).

A conversion of the hepatic adrenoceptor response analogous to that found in the 4-hr cells occurs in a number of conditions (see Introduction), and in some of these corresponding inverse changes in the density of α_1- and β-receptors have also been detected (12–14, 16, 36). Because these changes take days to develop *in vivo*, it is possible that the altered receptor numbers complement or follow corresponding inverse changes in the transduction mechanisms for the receptors, as suggested for the liver of adrenalectomized rats (36). In two recent reports, the density of [^{125}I]iodocyanopindolol-labeled β-receptors was found to increase severalfold within a few hours of primary culturing of adult rat hepatocytes (33, 34), which is in contrast to the present findings. However, cells in the above studies were cultured in a medium containing fetal calf serum and dexamethasone. This may have influenced receptor synthesis but is apparently not required for the conversion of the receptor response.

The reciprocal nature of the changes in α_1- and β-receptor

responses strongly suggests that regulation of the two receptor systems is closely coupled in the hepatocyte, at a level outside the ligand recognition sites. The two receptors may share a dissociable component ("holoreceptor"), or a common factor may be involved in the transduction of their signals. Although α_1- and β-receptors are believed to be coupled to distinct cellular pathways, there is some evidence to suggest the involvement of guanyl nucleotide binding proteins in the actions of both cAMP- and calcium-linked receptors. α_1-Receptor activation can increase cAMP levels in freshly isolated rat liver cells (18, 19) or in the hypothyroid rat myocardium (37), conditions associated with increased α_1-receptor dominance. Also, some data suggest guanyl nucleotide regulation of agonist binding to α_1-receptors (36). Furthermore, guanyl nucleotide binding proteins may be involved in regulation of calcium channels independent of their effect on adenylate cyclase (38). It may be proposed that changes in membrane phospholipase A_2 activity regulate the inverse coupling of α_1- and β-receptors to a guanyl nucleotide binding protein and thus control a link between cAMP- and calcium-dependent pathways of catecholamine action.

The mechanism of this phospholipase A_2 effect is not yet clear. The activity of this enzyme leads to the release of arachidonic acid, the common precursor of prostaglandins and leukotrienes, and also to the accumulation of lysophosphatidylcholine in the cell membrane. Phospholipase A_2 may influence the coupling of receptors directly, or indirectly through a prostaglandin or leukotriene intermediate. Alternatively, the accumulation of lyosphosphatidylcholine, a natural detergent, has been correlated with various changes in intracellular metabolism, which may affect receptor-effector coupling. Further work is needed to clarify these issues.

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