In Vivo Desensitization of Glycogenolysis to Ca^{2+}-mobilizing Hormones in Rat Liver Cells

Gozoh Tsujimoto,* Aiko Tsujimoto,† Kiyohiko Kato,‡ and Keitaro Hashimoto*  
Departments of *Pharmacology and †Pediatrics, Yamanashi Medical College, 1110 Tamaho-cho, Nakakoma-gun, Yamanashi, 409-38 Japan

Abstract

Rat hepatocytes contain several types of Ca^{2+}-linked receptors, all of which stimulate glycogen breakdown by increasing cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{c}). In vivo desensitization of this Ca^{2+} messenger system was studied in hepatocytes isolated from either pheochromocytoma (PHEO)-harboring and chronically noradrenergic (NE)-infused rats. Homologous desensitization for alpha,-adrenergic receptor-mediated phosphorylase activation was found in the early stage of PHEO rats (3–4 wk after implantation), whereas, in the later stage of tumor development or in the NE-infused rats, phosphorylase responses to all Ca^{2+}-mobilizing stimulations were subsensititious (heterologous desensitization). In the homologous desensitization, the [Ca^{2+}]_{c} response to alpha,-adrenergic stimulation was selectively reduced. We found, using the phenoxybenzamine inactivation method, that there was a linear relationship between alpha_{1} receptor density and the [Ca^{2+}]_{c} response; consequently, the blunt [Ca^{2+}]_{c} response to alpha_{1}-adrenergic stimulation could not be explained by the 34% downregulation of alpha_{1} receptors seen in these rats. These results indicated that uncoupling at a step proximal to alpha_{1} receptor-stimulated [Ca^{2+}]_{c} increase is also of primary importance in homologous desensitization of phosphorylase activation. On the other hand, heterologous desensitization also involved alteration(s) at steps distal to the rise in [Ca^{2+}]_{c}. Our data demonstrate that prolonged exposure to catecholamines results in desensitization of the [Ca^{2+}]_{c} mobilization pathway and may involve multiple mechanisms.

Introduction

Prolonged exposure of tissues to a variety of hormones or drugs often leads to a blunted response when the tissue is subsequently exposed to the agonist. This phenomenon has been called desensitization, tolerance, or tachyphylaxis. A great deal has been learned about the mechanism whereby receptor systems that activate adenylyl cyclase may desensitize (1, 2). For example, uncoupling and phosphorylation of beta-adrenergic receptors frequently have been found to be associated with the desensitization of adenylate cyclase activation that occurs after prolonged exposure to catecholamines.

Much less is known about desensitization of receptors that function through another important transmembrane signaling system, namely the phosphatidylinositol (PI) turnover/intracellular Ca^{2+}-mobilizing pathway. Alpha_{1}, adrenergic, vasoressin, and angiotensin II receptors are examples of receptors that function through this pathway and cause a rise of intracellular Ca^{2+} ([Ca^{2+}]_{c}) in a variety of cell types (for review see references 3 and 4). The increase in the [Ca^{2+}]_{c} that occurs when these receptors are activated may explain many of the physiological effects of these receptors. Several physiological and clinical examples exist that suggest that this signal transduction system also undergoes adaptive changes in the face of continued stimulation (5–9).

The liver is a major site of glycogen deposition and breakdown in the regulation of mammalian glucose homeostasis, and the balance between these processes is regulated by a variety of stimulators, including Ca^{2+}-mobilizing hormones. In particular, the regulation of glycogenolysis by Ca^{2+}-linked hormones has been extensively studied in adult male rat liver, in which glycogen phosphorylase, the key enzyme for this process, is mainly regulated by Ca^{2+}-mobilizing hormones. Its biochemical framework is also well established (for review see references 3, 4, 10, and 11); thus, the available experimental evidence indicates that an elevation of [Ca^{2+}]_{c} after the occupation of specific receptors by these Ca^{2+}-mobilizing hormones allosterically activates phosphorylase b kinase (12) and results in an increased rate of glycogen breakdown. Several in vitro studies involving an examination of desensitization of glycogen phosphorylase activation by Ca^{2+}-mobilizing hormones (13, 14) have used this model system of isolated rat hepatocytes. However, very little information is available on in vivo desensitization of responses mediated by Ca^{2+}-mobilizing hormones.

In these studies, we have investigated the consequences of prolonged exposure to catecholamines on the activation of hepatic glycogenolysis to Ca^{2+}-mobilizing hormones. High circulating levels of catecholamines were maintained in rats either by transplantable catecholamine-producing tumor pheochromocytoma (PHEO) or by subcutaneously implanted osmotic minipumps. We found that desensitization of hepatic glycogenolysis to Ca^{2+}-mobilizing hormones can occur in vivo settings. Experiments in hepatocytes isolated from rats chronically exposed to catecholamines revealed multiple mechanisms in the process of desensitization.
Methods

Materials

Chemicals were obtained from the following sources: Quin2-tetraacetoxyethyl ester (quin2/AM). Dojindo Laboratories, Kumamoto, Japan; type II collagenase, Worthington Biochemicals, Freehold, NJ; (+) phenylephrine HCl, angiotensin II, (±) propranolol HCl, Arg-vasopressin, glucagon, dibutyryl cAMP (Bt-cAMP), and Hepes, Sigma Chemical Co., St. Louis, MO; forskolin and A23187, Calbiochem-Behring Corp., La Jolla, CA. [3H]Prazosin (specific activity, 19.8 Ci/mmol) and alpha-D-[U-14C]Glucose-1-phosphate were from New England Nuclear (Boston, MA). Aequorin was purchased from Dr. J. R. Blinks of the Mayo Foundation (Rochester, MN) as 1 mg lyophilized protein in KCl and 1,4-piperazine-bis(ethanesulfonic acid) buffer. Phenoxylbenzamine HCl was generously supplied by SmithKline & French Laboratories (Philadelphia, PA). All other chemicals and reagents used were from standard commercial sources.

Animal model of PHEO

8-9 wk-old male New England Deaconess Hospital strain (NEDH) rats were fed standard laboratory chow ad lib, and maintained on a 12-h light/dark (0600/1800 h) cycle. Rats were lightly anesthetized with ether and implanted subcutaneously at the base of the neck with several pieces (£1 £1 x 3 mm) of rat PHEO taken from another NEDH rat harboring the tumor. Several tumor-bearing rats (line P-259 established by Warren and Chute, reference 15) were generously provided by Dr. William M. Manger, New York University Medical Center, New York, NY. Age-matched male NEDH controls were also lightly anesthetized with ether and received only an intrascapular skin incision. After the surgery, rats were housed in individual cages with free access to food and water. A palpable tumor mass was generally evident 3-4 wk after tumor implantation; the tumor was ~0.5-0.8 cm in diameter at this time. The animals' body weight also served as an indication of the progression of the tumor (15-17). Tumor-bearing animals gained weight at a rate similar to unimplanted age-matched controls for several weeks after tumor implantation. By the time the tumor was palpable, the body weight generally plateaued for 4-7 d and then rapidly decreased. The tumor-bearing animals used in this study exhibited body weight plateau at 21-28 d after tumor implantation. Animals were studied either when their body weight reached a plateau (early stage; 3-4 wk after tumor implantation) or when a rapid weight loss was apparent (late stage; 6-7 wk after tumor implantation); age-matched unimplanted controls were studied at the same time (17).

Infusion of norepinephrine (NE) in vivo

To examine whether the altered responsiveness observed in rats with PHEO was attributable solely to elevation of plasma NE or might be related to the presence of the tumor and associated changes in tumor-bearing animals, we compared changes using subcutaneously implanted osmotic minipumps to infuse NE. To raise circulating levels of NE in vivo, we implanted osmotic minipumps (Alzet; Alza Corp., Palo Alto, CA) subcutaneously on the backs of male NEDH rats. All rats used were 11-12 wk old and weighed ~280-300 g at the time of minipump implantation. The pumps, which are designed to infuse the drug continuously for up to 14 d at a constant rate of 500 µg/kg per h, were filled with NE in 0.001 N HCl; control rats were implanted with minipumps containing the vehicle (0.001 N HCl) alone. The rats were examined 24 h, 2 d, 4 d, or 7 d after implantation of the minipumps.

Analysis of plasma catecholamine concentrations

To analyze plasma catecholamine concentrations, blood (1 ml) from the inferior vena cava below the level of the renal veins was rapidly collected through an abdominal incision just before starting the liver perfusion. The blood was placed in ice-cold tubes containing 0.1% sodium EDTA as an anticoagulant. After centrifugation at 1,500 g for 10 min, the plasma samples were stored at ~80°C until analyzed. Plasma samples were extracted as previously described (18). Catechol-

amine determinations were made by reverse-phase HPLC with electrochemical detection as described previously (19) using the 88:12 (vol/vol) mixture of 0.1 M potassium phosphate buffer (pH 3.2) and methanol with 2.5 mM octane sulfonate sodium and 10 µM sodium EDTA as the mobile phase.

Preparation of hepatocytes

Isolation of hepatocytes was routinely initiated 3-4 h into the light period. Isolated hepatocytes were prepared by a modification (20) of the collagenase perfusion method of Berry and Friend (21) as described previously (22). Isolated hepatocytes from an individual rat were then divided for the measurement of phosphorylase a and [Ca2+]c, and for radioligand binding studies. The yield of cells from one liver averaged 1.5 x 10⁶, with 90-95% viability as estimated by trypan blue exclusion.

Fractional inactivation of alpha, receptors by phenoxylbenzamine

In some experiments to delineate the linkage between alpha,-adrenergic receptor occupation and [Ca2+]c response, we used the phenoxylbenzamine inactivation method (7, 23, 24). In these experiments, to irreversibly inactivate alpha,-adrenergic receptors, phenoxylbenzamine was added to the hepatocytes suspension in final concentrations ranging from 10⁻¹⁰-10⁻⁷ M and was incubated for 20 min at 37°C. Hepatocytes were then washed three times with the buffer used for the radioligand binding assay and for [Ca2+]c measurements. Data are expressed as percent of control; thus, for receptor occupation, B/Bmax, where B is the amount of [3H]prazosin specifically bound under the experimental conditions and Bmax is the amount of specific [3H]prazosin binding in the absence of any phenoxylbenzamine, and for [Ca2+]c measurements, as percent of the amplitude of aequorin bioluminescence response or that of peak [Ca2+]c measured by quin2 seen with 100 µM phenylephrine alone.

Analytical procedure

Glycogen phosphorylase a activity. Glycogen phosphorylase a activity was assayed in liver-cell homogenates prepared as described previously (22). Unless otherwise stated, cell samples were taken for determination of phosphorylase a activity 2 min after hormone additions. Glycogen phosphorylase a activity was measured using a filter disc assay similar to that of Gibboe et al. (25) as modified (26). Units of phosphorylase activity are micromoles of [14C]glucose from alpha-D-[U-14C]glucose-1-phosphate incorporated into total assay glycogen per min per 100 mg of protein.

Measurement of [Ca2+]c in rat hepatocytes. Measurement of [Ca2+]c in isolated hepatocytes was achieved by using either the photoprotein aequorin and the fluorophore quin2.

[Ca2+]c measurement (aequorin). Incorporation of aequorin into isolated hepatocytes was performed by the gravity loading method described by Borle et al. (27) with modifications. The aequorin-loaded cells were resuspended and incubated at 37°C in modified Hanks' buffer containing (in millimolar) NaCl, 137; KCl, 3.5; KH2PO4, 0.44; NaHCO3, 4.2; Na2HPO4, 0.33; CaCl2, 1.0; MgCl2, 1.0; glucose, 15; and 0.5% (w/v) dialyzed BSA. Bioluminescence was measured by a modified photomultiplier tube aggregometer (lumiaggregometer; Chrono-Log Corp., Haverton, PA) under constant stirring at 37°C. Resting [Ca2+]c was calculated from a calibration curve as previously published by Borle and Snowdowne (28, 29), assuming the even distribution of calcium in the cell. [Ca2+]c in stimulated cells was not calibrated, because no information was available as to the spatial distribution of calcium in the cell. The magnitude of aequorin luminescence is expressed as electric current. None of the drugs used in this experiment quenched luminescence of aequorin added to a cell-free suspension. Our preliminary experiments confirmed that there was no difference between aequorin-loaded and -unloaded hepatocytes in the glycogen phosphorylase responses induced by 100 µM phenylephrine, 10 µM epinephrine, 100 nM vasopressin, and 100 nM angiotensin II.
In some experiments, to determine the efficiency of aequorin incorporation, aequorin content was determined in an aequorin assay photometer by measuring the light signal emitted from lysed cells in the presence of a saturating concentration of CaCl₂ (28, 29). The resultant flash of light was recorded as current from the photomultiplier tube and normalized by the cell protein concentration.

\([\text{Ca}^{2+}]_\text{c} \text{ measurement (quin).}\] Quin2 loading was achieved essentially as reported by Thomas et al. (30). The cells were suspended in Hepes buffer containing (in millimolars) NaCl, 120; Hepes, 20; KCl, 5.4; NaHCO₃, 4.2; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 1.0; glucose, 15; and 2.0% (wt/vol) dialyzed BSA at pH 7.4 and maintained under an atmosphere of O₂/CO₂ (95:5%). After 20 min incubation with 100 μM quin2/AM, when maximal accumulation of quin2 had occurred, the cells were washed twice and resuspended, at ~3 mg of dry weight/ml in the Hepes buffer described above with the albumin concentration reduced to 0.2%. Quin2 fluorescence measurements were performed in a fluorescence spectrophotometer (model F-3000; Hitachi Ltd., Tokyo, Japan) equipped with a thermostat-controlled cuvette holder and magnetic stirring. Excitation and emission wavelengths were 340 and 490 nm, respectively. Calibration and determination of [Ca²⁺]c were done as described by Tsien et al. (31).

\([\text{³H}]\text{Prazosin binding.}\] [³H]Prazosin binding experiments were performed on a crude cellular homogenate as previously described (32). Saturation binding experiments with [³H]prazosin were carried out at a range of 0.5–5 nM.

Protein concentration was determined by the method of Lowry et al. (33) using BSA as standard.

Data analysis. All data for each drug were meaned and the resulting concentration-response curves from each group were analyzed simultaneously using the four-parameter logistic equation (34). The resulting ED₅₀ and maximal responses \( (E_{\text{max}}) \) were analyzed for significant differences using the ALLFIT program. The ALLFIT program is a modification of the DeLean et al. program by Martin H. Teicher, and was obtained from the Biomedical Computing Technology Information Center, Nashville, TN. Data derived from radioligand studies were analyzed by LIGAND, a nonlinear computer-assisted iterative weighted least square curve-fitting procedure (35).

The experimental data given in the text and figures are the means ±SEM of n experiments as indicated. Differences between means within each experiment were evaluated by analysis of variance. If analysis of variance demonstrated a significant difference among means, the t test for unpaired observations was then used to determine which pairs of means were significantly different.

Results

Effect of alpha₁-adrenergic stimulation on phosphorylase activity and \([\text{Ca}^{2+}]_\text{c} \text{ response.}\] Fig. 1 shows the time course of phosphorylase activation and \([\text{Ca}^{2+}]_\text{c} \text{ signals indicated either by quin2 or aequorin after addition of the alpha₁-adrenergic agonist phenylephrine (100 μM) to isolated hepatocytes. Significant phosphorylase activity was observed 5 s after phenylephrine addition, and this approached a maximum value after 15–20 s, which is kept by 3 min (Fig. 1 A). The time course of \([\text{Ca}^{2+}]_\text{c} \text{ signals seen in response to phenylephrine differed in aequorin- and quin2-loaded hepatocytes (Fig. 1, B and C). The aequorin signals displayed a more rapid rise in luminescence to a peak followed by a fall to baseline that may represent either decline in [Ca²⁺]c or aequorin consumption. However, the transient nature of the aequorin signal is not due to consumption of aequorin, since subsequent addition of a different agonist resulted in a prompt increase in the light; also, intracellular aequorin content measured by lysing the cells using Triton X-100 was not decreased significantly after a single addition of maximal dose of any of the agonists (data not shown). Quin2, on the other hand, demonstrated a similarly brisk upstroke to a peak, but followed by a plateau and a very slow decline in the signal, whose time course apparently corresponds well to that of phosphorylase activation.

In aequorin-loaded cells, resting [Ca²⁺]c of the rat hepatocytes was 148±16 nM \((n = 8)\), assuming intracellular magnesium concentration of 1 mM and even distribution of calcium in the cell. In quin2-loaded cells, 100 μM phenylephrine caused an increase in [Ca²⁺]c from a basal level of 175±14 nM \((n = 12)\) to a peak of 495±34 nM \((n = 8)\). In a series of preliminary experiments we observed an appropriate concentration-[Ca²⁺]c response relationship for several Ca²⁺-mobilizing hormones (phenylephrine, epinephrine, arg-vasopressin, and angiotensin II) in both aequorin luminescence and quin2 fluorescence. Also, using selective antagonists we observed that both aequorin- and quin2-induced [Ca²⁺]c responses to catecholamines occurred via alpha₁ receptors (data not shown).
Effects of receptor inactivation with phenoxybenzamine on \([^3H]prazosin binding and phenylephrine-stimulated \([Ca^{2+}]c\) response. After a 20-min incubation of freshly isolated hepatocytes with various concentrations of phenoxybenzamine, freshly isolated hepatocytes were extensively washed, and the extent of receptor inactivation was determined by \([^3H]prazosin binding to the remaining receptor sites. Maximum \([^3H]prazosin binding capacity was progressively decreased by exposure to increasing concentrations of phenoxybenzamine, reaching a maximal reduction of 94±3% at a phenoxybenzamine concentration of 10 \(\mu M\) (\(n=5\)), and the phenoxybenzamine concentration necessary to block half of the \([^3H]prazosin binding sites in 20\) min is 119±46 \(nM\) (\(n=5\)) (Fig. 2). Also, the effect of partial alpha\(_1\) receptor inactivation on 100 \(\mu M\) phenylephrine-stimulated \([Ca^{2+}]c\) response as assessed by quin2 and acuiror is shown in Fig. 2. The half-maximal inhibitory concentrations (IC\(_{50}\)) for 100 \(\mu M\) phenylephrine-stimulated \([Ca^{2+}]c\) response are 69±18 (\(n=5\)) and 78±58 \(nM\) (\(n=4\)) phenoxybenzamine for acuiror and quin2 signals, respectively. Correlation of fractional inhibition of phenylephrine-induced \([Ca^{2+}]c\) responses detected by acuiror and quin2 to fractional inhibition of \([^3H]prazosin binding approximated a unitary relationship in each case. The correlation of inhibition of phenylephrine-induced \([Ca^{2+}]c\) response detected by acuiror and \([^3H]prazosin binding has a slope of 1.13 and a correlation coefficient of 0.98. The slope of the line formed by a corresponding analysis of inhibition of phenylephrine-in-

**Table I. Comparison of Control and PHEO-implanted Rats**

<table>
<thead>
<tr>
<th></th>
<th>Control NEDH rats (11–12 wk old)</th>
<th>PHEO-bearing rats (Early stage)</th>
<th>Control NEDH rats (14–15 wk old)</th>
<th>PHEO-bearing rats (late stage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>288±4 ((n=16))</td>
<td>278±6 ((n=11))</td>
<td>348±3 ((n=14))</td>
<td>224±3* ((n=12))</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td></td>
<td>1.1±0.2 ((n=9))</td>
<td></td>
<td>5.3±0.4* ((n=8))</td>
</tr>
<tr>
<td>Plasma dopamine (pg/ml)</td>
<td>168±42 ((n=8))</td>
<td>33,300±9,200* ((n=7))</td>
<td>188±32 ((n=8))</td>
<td>41,600±12,700* ((n=8))</td>
</tr>
<tr>
<td>Plasma NE (pg/ml)</td>
<td>198±30 ((n=8))</td>
<td>28,400±5,440* ((n=7))</td>
<td>201±58 ((n=8))</td>
<td>22,300±4,720* ((n=8))</td>
</tr>
<tr>
<td>Plasma epinephrine (pg/ml)</td>
<td>128±22 ((n=8))</td>
<td>156±46 ((n=7))</td>
<td>108±29 ((n=8))</td>
<td>185±59 ((n=8))</td>
</tr>
</tbody>
</table>

* \(P < 0.01\) vs. age-matched control NEDH rats; **\(P < 0.01\) vs. early-stage PHEO-bearing rats; no symbol indicates no significant difference (\(P > 0.05\)). Plasma catecholamine measurements were done as described in Methods. Values are mean±SEM; \(n\) refers to the number of rats.

Desensitization of Intracellular \(Ca^{2+}\) Messenger System
Figure 3. (A) Activation of glycogen phosphorylase to phenylephrine (left) and epinephrine (right) in isolated rat hepatocytes. Three groups of animals were studied: age-matched controls, early stage, or late stage of PHEO-bearing rats. As no significant difference was obtained either between the younger and older age-matched controls or between the early and late stages of PHEO rats, these two groups of data were pooled. Each point with a vertical bar represents the mean±SEM of 12–15 experiments. (B) Activation of glycogen phosphorylase to Arg-vasopressin, angiotensin-II, and the Ca²⁺ ionophore A23187 in isolated rat hepatocytes. Three groups of animals were studied: age-matched control (○), early stage (▲), and late stage (△) of PHEO-bearing rats. Each point with a vertical bar represents the mean±SEM of five to seven experiments. (C) Activation of glycogen phosphorylase to glucagon, forskolin, and Bt₂cAMP in isolated rat hepatocytes. Three groups of animals were studied: age-matched control (○), early-stage (▲), and late-stage (△) of PHEO-bearing rats. Each point with a vertical bar represents the mean±SEM of at least four experiments. *P < 0.05 difference from controls.
loss in responsiveness to a multiplicity of agonists acting on different receptors (36). To address this issue, we have examined the phosphorylase activation to the two nonadrenergic hormones, arg-vasopressin and angiotensin II, and the Ca2+-ionophore A23187. As shown in Fig. 3B, in the early-stage PHEO rats, the Ca2+-mobilizing stimulus was similar to controls; whereas, in the late-stage PHEO rats, all concentrations examined the phosphorylase responses were significantly (P < 0.05) smaller compared with those of controls. Phosphorylase activation by stimuli that cause an increase in cAMP was also shown in Fig. 3C. In both early and late stages of PHEO rats, phosphorylase activity by Bt2cAMP and forskolin in these rats were not significantly different from those of controls, whereas glucagon-stimulated phosphorylase activation was found to be significantly lower in both stages of PHEO rats than in age-matched control rats.

In an effort to determine the mechanism for the desensitization of hepatic glycogen phosphorylase activation to Ca2+-mobilizing hormones in rats harboring PHEO, [Ca2+]c responses in hepatocytes to 100 μM phenylephrine, 10 mIU/ml arg-vasopressin and 10 nM angiotensin II were measured in both early and late stages of PHEO rats. Estimated resting [Ca2+]c values in the presence of 1 nM extracellular Ca2+ for hepatocytes of PHEO rats (aequorin-indicated [Ca2+]c were 181±26 nM, n = 8 and 197±31 nM, n = 7; quin2-indicated [Ca2+]c were 154±22 nM, n = 9 and 186±28 nM, n = 7) for the early and late stage of PHEO rats, respectively) were similar to those of age-matched controls (aequorin-indicated [Ca2+]c were 162±26 nM, n = 6 and 171±33 nM, n = 6; quin2-indicated [Ca2+]c were 197±34 nM, n = 5 and 178±30 nM, n = 5) for the early and late stage of controls, respectively. As shown in Fig. 4, both aequorin- and quin2-indicated [Ca2+]c signals induced by 100 μM phenylephrine were greatly decreased in the early stage of PHEO rats, and were abolished in the late stage of PHEO rats. However, the [Ca2+]c responses to arg-vasopressin and angiotensin II detected by both aequorin and quin2 methods were not significantly different from controls in the early stage of PHEO. With

![Graph](image)

Figure 4. Effects of PHEO on phenylephrine-, Arg-vasopressin-, and angiotensin II-induced changes in cytosolic free Ca2+ concentration in isolated rat hepatocytes. Three groups of animals were studied: age-matched controls (left), or early-stage (middle), late-stage (right); of PHEO rats in medium containing 1.0 mM extracellular calcium. Values shown are the mean±SEM (bars) of five to six experiments. *P < 0.01 difference from controls.
Experiments performed the early stage of pheochromocytoma-bearing rats (closed bar). Values shown are maximal number of receptor sites (mean±SEM). The affinities of alpha1-receptors for [3H]prazosin were not different in the two groups. *P < 0.05 difference from controls. (Right) Concentration-effect curves for inhibition of [3H]prazosin binding by (−) epinephrine in hepatocyte membranes from control (○) and the early stage of PHEO-bearing rats (●). The curves are the mean of five experiments performed in duplicate.

Figure 5. (Left) Changes in alpha1-adrenergic receptors in hepatocyte membranes from the age-matched control rats (open bar) and the early stage of pheochromocytoma-bearing rats (closed bar). Values shown are maximal number of receptor sites (mean±SEM). The affinities of alpha1-receptors for [3H]prazosin were not different in the two groups. *P < 0.05 difference from controls. (Right) Concentration-effect curves for inhibition of [3H]prazosin binding by (−) epinephrine in hepatocyte membranes from control (○) and the early stage of PHEO-bearing rats (●). The curves are the mean of five experiments performed in duplicate.

PHEO rats. This extent of increase was detectable by 14 h (the earliest time point tested) and remained at this level for the duration of the infusion (data not shown). Corresponding to the increase of NE, the body weight progressively decreased over the time of the infusion.

Hepatic phosphorylation was examined on 1–2, and 4–7 d of NE infusion. The basal level of the glycolen phosphorylase activity was slightly decreased in hepatocytes isolated from NE-infused rats. The activities were 0.97±0.20 U/100 mg protein (n = 11) for control, 0.68±0.18 (n = 8) and 0.78±0.21 (n = 11) U/100 mg protein for 1–2 d and 4–7 d of NE-infused rats, respectively. The activities of Ca2+-mobilizing hormones and cAMP-elevating agents to activate glycogen phosphorylase in control and NE-infused rats are presented in Fig. 6. In both groups of NE-infused rats, the phosphorylation activation to all Ca2+-linked stimulations was markedly decreased at all concentrations examined compared with the controls (Fig. 6, A and B); this attenuation was observed by 24 h of NE infusion, suggesting that the heterologous desensitization develops very rapidly by infusion of NE, in contrast to the situation in PHEO rats. In these rats, phosphorylase activations by Bt2cAMP and forskolin were not significantly different from those of controls; however, phosphorylase activation by glucagon was significantly (P < 0.05) lower than in control rats (Fig. 6 C).

Estimated resting [Ca2+]c values in the presence of 1 mM extracellular Ca2+ for hepatocytes of NE-treated rats (aequorin-induced [Ca2+]c were 202±21 nM, n = 5 and 218±31 nM, n = 8; quin2-induced [Ca2+]c were 184±29 nM, n = 5 and 197±47 nM, n = 5 for 1–2 days and 4–7 d infusion of NE, respectively) were not significantly different from those values of controls (aequorin-induced [Ca2+]c was 165±27 nM, n = 7; quin2-induced [Ca2+]c was 170±21 nM, n = 12). As shown in Fig. 7, 100 μM phenylephrine-induced changes in both aequorin- and quin2-induced [Ca2+]c are significantly (P < 0.05) reduced after 1–2 d of NE infusion and became nondetectable after 4–7 d of infusion. Similar to findings in the late stage of PHEO rats, rises in [Ca2+]c to arg-vasopressin and angiotensin II were found to be different between aequorin- and quin2-induced signals. In hepatocytes treated by NE infusion for 1–2 d, aequorin-induced [Ca2+]c signals were not significantly different from those in controls, whereas quin2-induced ones were significantly decreased. Longer infusion of NE (4–7 d) was found to result in a more general reduction in both aequorin- and quin2-induced [Ca2+]c signals; thus, quin2-induced rises in [Ca2+]c were not detectable in these cells.

We further examined the role of the alpha1 receptors in the rapid development of the reduction in [Ca2+]c response to alpha1-adrenergic stimulation observed in hepatocytes from NE-infused rats for 1–2 d. Saturation binding experiments performed with hepatocytes membrane preparations from rats with NE infusion (1–2 d) showed that the number of hepatic alpha1-adrenergic receptors was slightly decreased, but the observed decrease from control was not significant at the 5% level (98.3±10.6 fmol/mg protein, n = 5 in control rats vs. 79.5±9.6 fmol/mg protein, n = 5 in NE-infused rats), nor were the affinities of the alpha1-adrenergic receptors for [3H]prazosin in hepatic membranes different between control rats and rats with NE infusion (Kd = 0.17±0.05 mM, n = 5 in control rats vs. Kd = 0.13±0.02 mM, n = 5 in NE-treated rats).

Discussion

Using the glycogenolysis in the rat liver as a model system, we have examined in vivo desensitization of the Ca2+ messenger system. In both PHEO-harboring and chronically NE-infused rats, we found two different patterns of desensitization in the ability of Ca2+-mobilizing hormones to activate hepatic phosphorylase. In homologous desensitization for alpha1-adrener-

<table>
<thead>
<tr>
<th>Table II. Comparison of Control and NE-infused Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control NEDH rats (11-12-wk old)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Plasma dopamine (pg/ml)</td>
</tr>
<tr>
<td>Plasma NE (pg/ml)</td>
</tr>
<tr>
<td>Plasma epinephrine (pg/ml)</td>
</tr>
</tbody>
</table>

* P < 0.01 vs. age-matched control NEDH rats; *P < 0.05 vs. NE-infused (1–2 d) rats; no symbol indicates no significant difference (P > 0.05). Plasma catecholamine measurements were done as described in Methods. Values are mean±SEM; n refers to the number of rats.
Figure 6. (A) Activation of glycogen phosphorylase to phenylephrine (left) and epinephrine (right) in isolated rat hepatocytes. Three groups of animals were studied: age-matched controls (○), or 1–2-d NE-infused (●), and 4–7-d NE-infused (▲) rats. Each point with a vertical bar represents the mean±SEM of at least five experiments. (B) Activation of glycogen phosphorylase to Arg-vasopressin, angiotensin-II, and the Ca\(^{2+}\) ionophore A23187 in isolated rat hepatocytes. Three groups of animals were studied: age-matched controls (○), 1–2-d NE-infused (●), and 4–7-d NE-infused (▲) rats. Each point with a vertical bar represents the mean±SEM of five to seven experiments. (C) Activation of glycogen phosphorylase to glucagon, forskolin, and Bt\(2\)cAMP in isolated rat hepatocytes. Three groups of animals were studied: age-matched controls (○), 1–2-d NE-infused (●), and 4–7-d NE-infused (▲) rats. Each point with a vertical bar represents the mean±SEM of at least four experiments. *\(P < 0.05\) difference from controls.
gic receptor-mediated phosphorylase activation developed in the early stage of PHEO rats, downregulation of alpha, receptors and uncoupling at a step proximal to alpha, receptor-stimulated \([\text{Ca}^{2+}]_{c}\) increase were found. On the other hand, heterologous desensitization seen in the later stage of the tumor development or the NE-infused rats also involved alteration(s) at steps distal to the rise in \([\text{Ca}^{2+}]_{c}\).

Our study shows that the homologous desensitization of hepatic phosphorylase activation to alpha,-adrenergic stimulation seen in the early PHEO rats involves a marked alteration in the relationship between alpha, receptor occupancy and receptor-coupled \([\text{Ca}^{2+}]_{c}\) response. Under basal conditions, the relationship as defined by the phenoxybenzamine inactivation method was linear, indicating an absence of a receptor reserve in activating this intracellular event in isolated rat hepatocytes. This is in good agreement with previous observations by Lynch et al. (37) on the agonist concentration-response relationship in the same tissue; however, this situation seen in the liver is in sharp contrast to the nonlinearity between the same parameters in vascular smooth muscle cells, as shown by Colucci and Alexander (7). Based on the linear relationship in rat liver cells, the blunted \([\text{Ca}^{2+}]_{c}\) response to alpha,-adrenergic stimulation cannot be explained by the 34% decrease in alpha, receptor number that was actually detected. Therefore, uncoupling in receptor occupancy-\([\text{Ca}^{2+}]_{c}\) response at a step proximal to \([\text{Ca}^{2+}]_{c}\) mobilization and downregulation of receptors, are involved in the process of this type of desensitization. Furthermore, in the desensitization that developed in the NE-infused rats within 1–2 d, the phenylephrine-induced \([\text{Ca}^{2+}]_{c}\) response was found to be significantly reduced without downregulation of alpha, receptors. This observation further supports the hypothesis that an alteration in receptor occupancy-\([\text{Ca}^{2+}]_{c}\) response coupling, rather than downregulation, is of primary importance in the process of desensitization at a step proximal to \([\text{Ca}^{2+}]_{c}\) mobilization.

The exact biochemical mechanism for this uncoupling of alpha, receptors is uncertain from this study, especially since our radioligand binding study could not detect any alteration in the binding properties of alpha, receptors for \(^{3}H\)prazosin in the desensitized hepatocytes. Using a different model system of smooth muscle cells, this functional uncoupling of alpha,-adrenergic receptors to the PI turnover/\([\text{Ca}^{2+}]_{c}\) mobilization pathway has been well studied (6, 7). We previously found that in vitro, 7 h exposure of rabbit aorta to alpha,-adrenergic agonists leads to homologous desensitization of alpha,-receptor-mediated smooth muscle contraction. This desensitization of contraction was not due to a change in alpha,-adrenergic receptor number or affinity for agonists, but there was a blunting of NE-stimulated PI turnover (6). Similar results have been confirmed using cultured vascular smooth muscle cells from rabbit aorta (7). Furthermore, Leeb-Lundberg et al. recently found that functional uncoupling of alpha,-adrenergic receptor-mediated PI metabolism in transformed smooth muscle cells (DDT1 MF-2) is closely related with the phosphorylation of the Mr 80,000 alpha,-adrenergic receptor ligand binding peptide, but the phosphorylation is without effect on its binding properties (38). Thus, the covalent modification of plasma membrane receptors by phosphorylation seems to play an important role in the desensitization of only receptors that couple with adenylate cyclase but also those that couple to PI turnover/\([\text{Ca}^{2+}]_{c}\) mobilization pathway. Further studies are clearly required to explain whether the possible phosphorylation of receptors by these mechanisms is causally related with the uncoupling seen in the in vivo homologous desensitization of hepatic phosphorylase responses.

To analyze the mechanism for the heterologous desensitization, it is important to determine first which \([\text{Ca}^{2+}]_{c}\) signal being investigated better reflects the physiologically important \([\text{Ca}^{2+}]_{c}\) change, since there was an apparent disparity between aequorin and quin2 results in the hepatocytes exerting heterologous type of desensitization. We have used both the fluorophore quin2 and the \(\text{Ca}^{2+}\)-sensitive photoprotein aequorin in this study, as these indicators have been reported to reflect different aspects of \(\text{Ca}^{2+}\) homeostasis; aequorin may be more sensitive to local and low levels of \(\text{Ca}^{2+}\) transients, whereas quin2 may respond more to the average or diffuse \([\text{Ca}^{2+}]_{c}\) (39). Characterizing both aequorin- and quin2-induced \([\text{Ca}^{2+}]_{c}\)
signals in rat hepatocytes, we found that they were generally similar in their nature, except for the time-course of the response. Aequorin-indicated response shows a very transient rise that returns to the baseline within 1–2 min, whereas the quin2-indicated one is more sustained and apparently well correlated with the changes in phosphorylase α. The similarity in the time course, however, does not necessarily mean that the quin2 response is better correlated with functionally important changes in [Ca\(^{2+}\)]c. For example, in platelets where the same situation exists for the time course of changes in [Ca\(^{2+}\)]c and functional correlates, aequorin-indicated [Ca\(^{2+}\)]c appears to be more closely related to both stimulatory and inhibitory changes in shape change, aggregation, or secretion than are rises in the quin2-indicated [Ca\(^{2+}\)]c (40, 41). Thus, from the present characterization it is hard to determine which signal better reflects the [Ca\(^{2+}\)]c: change responsible for phosphorylase activation in hepatocytes.

With the difficulty in interpreting the [Ca\(^{2+}\)]c results, heterologous desensitization seems to be a complex process and cannot be completely explained solely by alterations at a step proximal to [Ca\(^{2+}\)]c mobilization. This type of desensitization of hepatic phosphorylase has been reported in several in vitro studies (13, 14). In one study, attenuation of agonist-promoted phosphorylase activity was observed to be paralleled by a decreased ability of the hormones to stimulate hydrolysis of inositol phospholipids, suggesting that the desensitization of glycogen phosphorylase activation involves unknown events that couple PI breakdown to intracellular Ca\(^{2+}\) mobilization (14). As shown in these in vitro studies and the homologous desensitization seen in the early stage of PHEO rats, regulation at the level of the receptor-[Ca\(^{2+}\)]c response may represent one important group of adaptive responses directed at dampening excess stimulation, but these mechanisms do not exclude the role of modification of other cellular mechanisms in desensitization. Our data in the tolerant hepatocytes support the possibility that the in vivo development of this type of desensitization can be accomplished by a mechanism distal to [Ca\(^{2+}\)]c mobilization as well, especially since phosphorylase activation by the Ca\(^{2+}\) ionophore A23187, which bypasses the receptor-mediated [Ca\(^{2+}\)]c mobilization, is blunted in hepatocytes that exert heterologous type of desensitization.

The data indicate a near total loss of phosphorylase activation to all Ca\(^{2+}\)-linked stimulation after NE infusion or prolonged exposure to pheochromocytoma. Even in these tolerant hepatocytes CAMP-dependent phosphorylase activation by forskolin and Bt2CAMP remains relatively unaffected, suggesting the defect is confined to Ca\(^{2+}\)-mobilizing pathway. However, the ability of glucagon in stimulating phosphorylase was found to be significantly decreased in these cells. These results indicate the site of the lesion for the reduced glucagon-stimulated response may lie at the level proximal to CAMP generation. Similar observations were also made in vitro (42), suggesting that specific desensitization of glucagon-stimulated adenylate cyclase can be elicited by hormones that stimulate the breakdown of inositol phospholipids and Ca\(^{2+}\) mobilization. Another important consideration with respect to the attenuated glucagon-stimulated phosphorylase activation in the tolerant hepatocytes is the possible existence of glucagon-stimulated PI turnover/Ca\(^{2+}\) mobilization pathway. The phosphorylase activation by glucagon is now suggested to be mediated by two distinct population of receptors, each couples with different second-messenger systems; one set is linked to stimulate PI turnover/Ca\(^{2+}\) mobilization pathway (GR1 receptors), and the other set being presumed to account for the stimulation of adenylate cyclase activity (GR2 receptors) (43). Consequently, it is possible that not only glucagon GR2 receptor-stimulated CAMP production system but also GR1 receptor-mediated PI/Ca\(^{2+}\) mobilization pathway itself is undergoing desensitization in these cells. These possibilities are now under investigation.

Desensitization was found in the ability of Ca\(^{2+}\)-mobilizing hormones to activate hepatic phosphorylase in both rats harboring PHEO and NE-infused rats. However, the picture and time course of desensitization was markedly different between these two models. The explanation for the differences between two animal models is unclear. Possible factors that could relate to this disparity involve the different NE delivery rate by the tumor from that by osmotic minipump; thus, plasma NE in the PHEO rats was reported to rise gradually to maximum levels by 4 wk after tumor transplantation (44), whereas plasma NE levels were elevated ~ 30-fold by 14 h, the earliest time tested, and remained at this level for all 7 in the rats implanted with osmotic minipumps. Also, the potentially important role of the markedly elevated dopamine concentration in the tumor-bearing rats should be considered. Dopamine has been known to be a partial agonist at alpha-adrenergic receptors (45). Partial agonists may antagonize the effects of the endogenous full agonist, NE. Moreover, recent studies have revealed that PHEO may produce and secrete various neuropeptide-like substances besides catecholamines (46). Thus, this multisecretory nature of this amine-precursor uptake decarboxylation tumor could contribute the disparity observed. Whatever the explanation, the current results emphasize that one must be careful in extrapolating findings obtained for desensitization of adrenergic receptor functions in the PHEO model to other settings. The similar observation that increases in catecholamines by implantation of PHEO or infusion of agonists produce somewhat different patterns of regulation of adrenergic receptors has been documented previously in rat renal cortex (47).

In conclusion, our results indicate the complexity and diversity of mechanisms that may mediate desensitization to excessive in vivo stimulation of Ca\(^{2+}\)-messenger system. Not only may desensitization occur through functional uncoupling and downregulation of receptors but also postreceptor mechanism(s), including distal to [Ca\(^{2+}\)]c mobilization may be involved in certain cells. Hepatocytes may be a useful model system to pursue further these more poorly understood possibilities.

Acknowledgments

The authors gratefully acknowledge Dr. William M. Manger (New York University of Medical Center) for generously providing us several tumor-bearing NEDH rats, Dr. Akemi Minegishi (National Medical Center, Tokyo) for measurement of plasma catecholamines, and Dr. Shoji Kume (Department of Clinical Laboratory, Yamanashi Medical College) for the loan of the lumiaggregometer. We particularly thank Dr. Brian B. Hoffman (Department of Medicine, Stanford University Medical School, Palo Alto, CA) for his valuable comments and continuing encouragement. Ms. Reiko Mitsui provided excellent technical assistance and Ms. Mie Yamada excellently prepared the manuscript.

G. Tsuchimoto is a recipient of a Young Investigator Development Award of Meiji Life Foundation and Uehara Memorial Foundation. This work was supported in part by a research grant from the Scientific
References


39. Johnson, P. C., J. A. Ware, P. B. Cliveden, M. Smith, A. M.


