Modulation of glucagon receptor expression and response in transfected human embryonic kidney cells

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Ikegami, Tadashi, Aaron M. Cypess, and Bernard Bouscarel. Modulation of glucagon receptor expression and response in transfected human embryonic kidney cells. Am J Physiol Cell Physiol 281: C1396–C1402, 2001.—The modulation of glucagon receptor (GR) expression and biological response was investigated in human embryonic kidney cell (HEK-293) clones permanently expressing the GR with different densities. The GR mRNA expression level in these clones was upregulated by cellular cAMP accumulation and presented a good correlation with both the protein expression level and the maximum number of glucagon binding sites. However, the determination of glucagon-induced cAMP accumulation in these cell lines revealed that the enhancement of receptor expression did not lead to a proportional increase in cAMP formation. Under these conditions, the maximum cAMP production induced by NaF and forskolin was not significantly different among selected clones, regardless of the receptor expression level. High receptor-expressing clones showed the greatest susceptibility for agonist-induced desensitization compared with clones with lower GR expression levels. The results of the present study suggest that the GR can recruit non-GR-specific desensitization mechanism(s). Furthermore, the partial inhibition or alteration of the overall cAMP synthesis pathway at the receptor level may be a necessary adaptive step for a cell in response to a massive increase in membrane receptor expression level.

G protein-coupled receptor; adenosine 3',5'-cyclic monophosphate; desensitization; HEK-293 cells

ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE (cAMP) is widely recognized as a second messenger that regulates intracellular metabolism and cell proliferation and differentiation, as well as gene expression in mammalian cells. A large number of neurotransmitters and hormonal agonists act through membrane receptors coupled to heterotrimeric GTP-binding (G) proteins to regulate adenyl cyclase (AC) activity and cAMP synthesis.

Glucagon is specifically processed from proglucagon in the pancreatic α-cells and is secreted in response to low blood glucose levels. Through cAMP production, one of the main physiological functions of glucagon is to maintain glucose homeostasis, primarily by both stimulating glycogenolysis and gluconeogenesis and by inhibiting glycolysis (14). Recent reports showed that the glucagon receptor (GR) signaling pathway is altered in certain diseases such as hepatic cirrhosis in humans (6) and cholestatic liver disease in an animal model (15). The clarification of the mechanism responsible for this alteration would increase the understanding of the complications of cirrhosis and aid in developing novel methods to improve long-term survival and quality of life for the patients.

The GR is closely related to the glucagon-like peptide-1, secretin, vasoactive intestinal peptide, and gastric inhibitory polypeptide receptors, and belongs to the superfamiliy of G protein-coupled receptors (GPCRs) (13). To achieve its intracellular effects, glucagon must bind to a GR, which has seven putative transmembrane domains. Although the regulatory mechanism of GR expression and its structure have been studied intensively in the past 5–10 yr (1, 8), few studies have focused on the complex GR/G protein/AC, despite the physiological importance of the GR signaling pathway. Manipulation of the cellular GR expression in relation to the glucagon-induced signal transduction will provide information critical to the understanding of impaired glucagon signaling response under pathological conditions.

As a part of this work, the rat GR cDNA was expressed in human embryonic kidney cells (HEK-293) under the control of the cytomegalovirus (CMV) promoter/enhancer system (9). We have subcloned this cell line according to the steady-state expression level and biological activity of GR. In this study, we show that agents that stimulate cAMP production upregulate the GR expression level. In addition, this compulsory expression led to greater susceptibility for receptor desensitization and lowered responsiveness to agonist stimulation of cAMP production.

MATERIALS AND METHODS

Cell culture. HEK-293 cells were transfected with the rat GR (HEK-GR) (9). This cell clone was established by transfection with the synthetic rat GR cDNA (1.5 kb) inserted into...
a eukaryotic cell expression vector, pcDNA3 (Invitrogen, Carlsbad, CA). The HEK-GR cells were maintained in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G and streptomycin, 10 mM HEPES (pH 7.2), l-glutamine, and nonessential amino acids at 37°C in a humidified atmosphere of 95% air-5% CO₂.

Identification of GR-expressing clones. For cloning, the HEK-293 cells were suspended and diluted at a concentration of 10 cells/ml in culture medium, and each well of a 96-well plate was inoculated with 100 μl of cell suspension. Only wells having a single cell were selected microscopically and cultured. HEK-293 cell clones were dissolved in 500 μl of a 3 M guanidium-thiocyanate solution containing 50% DMSO, 12.5 mM sodium citrate, and 0.125% sarkosyl. Cell lysates were denatured by incubation at 65°C for 1 h and applied to a dot-blotting apparatus (Bio-Dot Apparatus; Bio-Rad, Hercules, CA) assembled with a nylon membrane (Hybond-N; Amersham Pharmacia, Piscataway, NJ). The blotted membrane was rinsed with 20× saline sodium citrate (SSC) and applied for hybridization as described below.

Total RNA was obtained from cells incubated in 60-mm culture plates by the method of Chomczynski and Sacchi (7) with minor modification using RNA-Zol B reagent (Tel-Test, Friendswood, TX). Ten micrograms of denatured total RNA were electrophoresed in 1× MOPS/EDTA buffer on a 1.2% agarose gel. RNA was transferred to a nylon membrane in 20× SSC and immobilized by ultraviolet cross-linking. The RNA was hybridized with a randomly [32P]dCTP-labeled cDNA probe for rat GR or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. Hybridization was carried out in Hybrisol I (Intergen, Purchase, NY) at 42°C for 8–16 h. The membrane was then washed twice with 2× SSC/0.5% SDS for 30 min (25°C) and twice with 0.2× SSC/0.5% SDS for 30 min each (65°C). After overnight exposure to a Phosphor-Imager screen, the membrane was scanned and analyzed using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Except where otherwise indicated, the GR mRNA was quantitated relative to that of GAPDH. Western blot, ligand binding assay, and cAMP determination were performed as previously described (4, 15).

Membrane preparation and determination of AC activity. Cell membranes from the HEK-293 clones were prepared by passage 10 times through a 26-gauge needle in ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 20 μg/ml leupeptin]. Nuclei and undisrupted cells were sedimented at 500 g for 5 min. The supernatants were centrifuged at 50,000 rpm for 30 min in a Beckman TL100-3 centrifuge (Beckman, Palo Alto, CA) and washed with lysis buffer twice. AC activity was determined by measuring the production of cAMP from [α-3H]ATP as described by Salomon et al. (19). In these experiments, [3H]cAMP was used to correct for differences in recovery rate.

Statistical analyses. Except where otherwise indicated, results were expressed as means ± SE. The statistical significance of the mean was determined by either one-way analysis of variance or Student’s paired t-test.

RESULTS

To identify the GR-expressing cell (HEK-GR) clones, >40 clones were screened by dot blotting using radiolabeled GR cDNA as a probe. The steady-state mRNA expression levels of GR, quantified as a ratio to those of GAPDH, ranged from 34:1 for the maximum (Fig. 1, clone 1-4B) to <0:6:1 for the minimum (clone 2-5D).

Among 10 clones selected, the expression level of GR mRNA determined by Northern blotting is shown in Fig. 1. Clones 2-5D, 2-10F, 4-4E, and 5-5D had low or no detectable GR mRNA level, and clone 2-5D was selected as a control for the subsequent studies. In addition, one highly (1-4B) and three moderately (2-2C, 2-10E, and 3-8D) expressing clones were also selected. The GR mRNA expression level among the different clones was well conserved over at least 10 passages. The proliferation rate for each clone was determined by successive cell number counting and calculation of doubling time. There were no significant differences among the doubling times for each clone (average 23.9 h).

The effects of various cAMP synthesis stimulatory agents were tested on the GR mRNA expression level. The expression level of the GR mRNA in clone 2-2C was upregulated by ~70% 24 h after incubation of cells with glucagon at the concentration of either 1 μM (Fig. 2A, lanes 3 and 4) or 100 nM (Fig. 2B, lane 4) in the presence of IBMX. Similar upregulation of the GR mRNA expression was observed with either 10 μM forskolin (Fig. 2B, lane 2), 100 μM 8-bromo-cAMP (lane 3), or 5 μM isoproterenol (lane 5) in the presence of 100 μM IBMX (Fig. 2B). To support a glucagon-induced cAMP-dependent regulation of the GR mRNA expression level in our clones, we incubated the cells with either 5 or 20 μM of H-89 (Fig. 2C, lanes 4 and 5), a known cAMP-dependent protein kinase inhibitor, and determined the GR mRNA level 24 h after addition of

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**Fig. 1.** Identification of human embryonic kidney (HEK-293) cell glucagon receptor (GR) clones. Total cellular RNA was isolated from each clone, loaded (10 μg/lane) on a 1.2% agarose gel, and transferred onto a nylon membrane. A: the membrane was probed sequentially with the GR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. B: the GR mRNA expression level was quantitated relative to the respective GAPDH mRNA expression level. Results are the average of 2 separate experiments from 2 different passages.
mRNA induced by glucagon from 30% with a dependent manner, the increased expression of the GR.

100 nM glucagon (Fig. 2C). H-89 inhibited, in a dose-dependent manner, the increased expression of the GR mRNA induced by glucagon from 30% with 5 μM to >80% with 20 μM. However, this inhibitor did not significantly inhibit either the basal GR or the GAPDH mRNA level (Fig. 2C, lane 2). These levels of GR mRNA, as modulated by cAMP synthesis stimulatory agents, were seen among all selected clones except 2-5D, which had no detectable GR mRNA. The possible involvement of either growth factors or other hormones in this GR mRNA expression level was excluded by observing similar upregulation of GR mRNA expression 24 h after incubation of the cells with glucagon and in the presence and absence of FBS.

To assess whether the cAMP-induced upregulation was due to modulation of the GR gene transcription, HEK-GR cells were cultured with 4 μg/ml actinomycin D (AMD), which is known to be a strong inhibitor of gene transcription. AMD suppressed the induction of the GR mRNA expression by glucagon (Fig. 3). This inhibition was gene specific and was not due to a toxic effect of this drug, since under the same conditions, the GAPDH mRNA expression level was unaffected.

As shown in Fig. 4A, the GR protein expression level in each selected clone was determined by Western blotting using a specific polyclonal antibody raised against the synthetic rat GR peptide. The protein expression level presented a significant correlation with that of the GR mRNA (r = 0.974, P = 0.0002).

The binding of 125I-glucagon to membranes from the different HEK-GR clones is shown in Fig. 4B. Membranes from HEK-GR were incubated with radiolabeled glucagon and increasing concentrations of unlabeled glucagon. The required concentration of unlabeled glucagon to inhibit the 125I-glucagon binding by 50% (IC50) was similar among the clones tested with an average value of ~23 nM, as determined from the fit of the respective sigmoidal curve. These values were in the same range as that previously reported for the GR cloned in COS-1 cells. However, under these conditions, the maximum number of glucagon binding sites varied from a minimum of 534 fmol (clone 2-10E) to a maximum of 996 fmol (clone 1-4B). It should be mentioned that the use of the agonist to determine the glucagon binding characteristics may lead to an underestimation of the maximum binding. The number of GR receptor binding sites presented a significant correlation with both the GR mRNA and protein expression level for each respective clone (binding site vs. mRNA: r = 0.868, P = 0.005; binding site vs. protein: r = 0.902, P = 0.005).

The basal cellular cAMP concentration, determined by radioimmunoassay, was not significantly different among the different HEK-GR clones. The cellular cAMP production was studied after 15 min of incubation with increasing concentrations (10−11−10−7 M) of glucagon in the presence of IBMX. When expressed per milligram of protein, while the concentration necessary to elicit 50% of the maximum effect was similar among the clones (EC50 ~ 7.0 nM), the maximum cAMP synthesis varied from a minimum of 1,607 fmol/mg protein for clone 1-4B to a maximum of 3,735 fmol/mg protein for clone 3-8D. It should be mentioned that the use of the agonist to determine the glucagon binding characteristics may lead to an underestimation of the maximum binding. The number of GR receptor binding sites presented a significant correlation with both the GR mRNA and protein expression level for each respective clone (binding site vs. mRNA: r = 0.868, P = 0.005; binding site vs. protein: r = 0.902, P = 0.005).

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The glucagon-induced cAMP production did not parallel that of either the expression level of the GR mRNA or the maximum number of glucagon binding sites. The femtomole of cAMP produced per femtomole of GR, i.e., receptor density, varied from 1.6–6.2 (Table 1).

To assess whether other sites between the receptor and the AC could be altered and, therefore, responsible for the differential induction of cAMP by glucagon between clones, we compared the effect of NaF and forskolin, known to directly activate the G protein and AC, respectively, on the cell membrane AC activity and cAMP synthesis. As shown in Fig. 6, 20 mM NaF and 100 μM forskolin stimulated AC activity approximately two- and sixfold, respectively, without significant differences among the selected clones, whereas under these conditions (Fig. 6B), there was a differential stimulatory effect of glucagon on AC activity between clones 1-4B and 3-8D. Ten nanomolar glucagon maximally activated AC in both clones. However, while concentrations of glucagon >10 nM activated AC by approximately threefold in clone 1-4B, it increased to approximately fivefold in clone 3-8D. These results are in agreement with those of glucagon-induced cAMP production reported in these clones (Fig. 5).

The membrane-associated GR expression level was increased over time with a maximum increase observed 24 h following the addition of 1 μM glucagon to the culture medium in both 1-4B and 3-8D clones (Fig. 7A). In addition, these newly expressed receptors were able to bind glucagon with a similar affinity (data not shown). The respective maximum glucagon binding for clones 1-4B and 3-8D was 6.1 ± 1 pmol/mg of protein and 5.4 ± 1.5 pmol/mg of protein and was not significantly different in the absence and presence of glucagon for 4 h (Fig. 7B). However, the maximum glucagon binding increased by 46% and 270% to 15.1 ± 1.6 pmol/mg of protein and 20.4 ± 5.1 pmol/mg of protein for clones 1-4B and 3-8D, respectively, after 24 h of incubation with glucagon. This increased membrane GR expression level, and maximum glucagon binding did not result in a parallel increased ability for glucagon to stimulate the AC (Fig. 7C). Furthermore, the 1-4B clone completely lost the AC responsiveness to glucagon 4 h after treatment with the agonist, and this decrease was still noticeable 24 h after glucagon treatment. This phenomenon was much more limited with clone 3-8D (Fig. 7C). In this clone, the decreased acti-

Fig. 4. Respective GR expression level and glucagon binding specificity in the selected HEK-GR clones. For protein expression level, membrane fractions from the selected HEK-GR clones (10 μg of protein) were loaded onto a 9% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto a nitrocellulose membrane. A: the membrane was immunoblotted using primary antibody directed against either rat GR or β-actin and a horseradish peroxidase-labeled secondary antibody. For glucagon binding assay, competitive displacement of 125I-labeled glucagon bound to the selected HEK-GR clones was determined by incubating 25 μg of protein/assay with 1 nM of 125I-glucagon and increasing concentrations (10^-11–10^-6 M) of unlabeled glucagon. B: data are presented as the total binding of the radiolabeled glucagon expressed in disintegrations per minute vs. the log of the glucagon concentration. Each value represents the mean of triplicate determinations and was best fitted by a single ligand-binding site model. SE was <10% of the mean and was omitted for better clarity of the graph.

Fig. 5. HEK-GR clones were incubated with 100 μM IBMX and increasing concentrations (10^-11–10^-7 M) of glucagon for 15 min. At the end of this period of incubation, the reaction was stopped by the addition of 24% HClO4, and the total cellular cAMP concentration was determined by RIA. Results of the total cAMP production are means ± SE from 3 experiments assayed in duplicate and are expressed as femtomoles of cAMP per milligram of protein. The respective basal cellular cAMP production was 11.7, 7.6, 9.2, 14.1, and 10.2 fmol/mg of protein for clones 1-4B, 2-2C, 2-10E, 3-8D, and 2-5D.
vation of AC following incubation of cells with glucagon was significant only after 4 h and disappeared after 24 h. The respective recovery of the glucagon signal after 24 h of incubation is probably due to the limited stability of the hormone. However (not shown), the glucagon-induced cAMP production returned to control levels for both clones after 48 h of incubation with glucagon.

**DISCUSSION**

The diversity of expression level of transfected gene products in different clones can be the result of a single transfection procedure. In the present study, the steady-state GR mRNA expression levels varied among selected HEK-293 clones. The mRNA and protein expression levels increased in a parallel manner up to two- and fourfold, respectively. Furthermore, while the maximum number of binding sites differed from clone to clone, the affinity of the receptor for glucagon was not significantly affected and was in the same range as what has previously been reported in the liver and in GR-transfected cell lines (3, 9). Therefore, it can be concluded that the overexpression of GR in HEK-293 cells does not affect the receptor-ligand interaction.

In the present study, HEK-293 cells, which do not naturally possess GR, were transfected with the coding domain of the rat GR gene ligated into the expression vector pcDNA3 (9). One of the original components of the pcDNA3 vector, the CMV promoter, was employed as a promoter for the constitutive expression of GR. This transfected HEK-293 cell line showed a glucagon-induced, increased GR mRNA transcription, at least in part, in a cAMP-dependent manner. Several groups have suggested that the basal activity of a major immediate early enhancer of human CMV was cell cycle dependent (5) and could be augmented considerably by elevated levels of intracellular cAMP in a cell type-specific manner (20). Therefore, according to the result of the consensus sequence search, since the CMV promoter contains several activator protein-1 transcription factor sites as well as the cAMP-response element, the positive transcriptional regulation by cAMP is one of the possible explanations for the upregulation of GR mRNA following cAMP production in this system.

There was no correlation between the steady-state number of GRs and the associated cAMP production in the present study. Indeed, the clone with the highest receptor density (1-4B) was the least effective, as far as cAMP production was concerned. The relationship between receptor number and the associated cAMP production has been previously discussed, mainly for the β-adrenergic receptor (β-AR) (18). In both cultured myocytes and transgenic mice, overexpression of the β-AR protein 20- to 200-fold resulted in a <2-fold increase in isoproterenol-induced cAMP production (10, 17). The conclusion from these studies was that the AC expression level was the limiting factor in the receptor-mediated cAMP production (12, 18). In the present study, it is supposed that the AC activity itself

### Table 1. Characteristics of glucagon response in the selected HEK-GR cell clones

<table>
<thead>
<tr>
<th>Clones</th>
<th>Binding Characteristics</th>
<th>cAMP Production</th>
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<tbody>
<tr>
<td></td>
<td>IC50, nM</td>
<td>Receptor density, fmol</td>
</tr>
<tr>
<td>1-4B</td>
<td>16.2</td>
<td>996*</td>
</tr>
<tr>
<td>2-2C</td>
<td>25.4</td>
<td>596</td>
</tr>
<tr>
<td>2-10E</td>
<td>17.3</td>
<td>534</td>
</tr>
<tr>
<td>3-8D</td>
<td>33.4</td>
<td>764*</td>
</tr>
<tr>
<td>2-5D</td>
<td>10.2</td>
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Values presented were derived in part from Figs. 4 and 5 by a nonlinear least-squares regression analysis. Binding characteristics and cAMP production are the averages from 3 independent experiments performed in triplicate. Maximum cAMP production was induced by glucagon. *P < 0.005 vs. clone 2-10E, †P < 0.005 vs. clone 1-4B.
was identical between each clone since forskolin-induced cAMP production was not different between selected clones having different GR expression levels. Furthermore, the glucagon-induced maximum cAMP production was <80% of that induced by 100 μM forskolin, thus eliminating in our model any suggestion of the AC as a limiting factor.

The glucagon-induced complete inhibition of the glucagon response, also named homologous desensitization (2), persisted for up to 24 h in the 1-4B clone with a high GR expression level and modest glucagon responsiveness. Glucagon had a more limited desensitization effect in the 3-8D clone, with a more modest GR expression level and high glucagon responsiveness (Fig. 7B). The loss of hormonal responsiveness in GPCRs induced by the agonist can be observed in seconds to minutes following ligand binding and can be mediated by various mechanisms including activation of GPCR kinases (see Ref. 11 for review). The ability of cAMP to stimulate GR mRNA and protein expression in the present study excludes any possible transcriptional downregulation of GR in our system (Fig. 7A). In addition, since there was no significant change in either forskolin- or NaF-induced AC activity in selected clones (Fig. 6), this loss of responsiveness exclusively implicates the GR. Therefore, we speculate that this greater susceptibility of the receptor to desensitization might be an adaptive step for a cell in response to a massive increase in membrane receptor expression level. Our hypothesis is supported by the conclusion of Bohm et al. (2) that this desensitization mechanism is important to prevent any uncontrolled GPCR stimulation of signaling pathways. Furthermore, our findings underline a broad compatibility of the desensitization system for the GR even if this receptor is not naturally expressed in the cell. Finally, these clones will be useful tools to clarify the molecular mechanism(s) of GR desensitization.

This adaptation of the overall cAMP synthesis cascade to a net increase in receptor is physiologically
relevant and can, to a certain extent, explain the results reported by Michel et al. (16). These authors observed in the spontaneously hypertensive rat, and as a function of age, that while the renal β-AR number was significantly increased, the associated cAMP production remained unchanged. Furthermore, under these conditions the forskolin-induced cAMP production remained unaffected and was 10-fold greater than that induced by the adrenergic receptor. Together, these results suggest that under pathophysiological conditions the net increase in receptor number is not always consequently associated with an increased cAMP production.

In conclusion, the differential response of desensitization can explain, at least in part, the lack of a relationship between receptor number and hormonal responsiveness in these clones. It is also possible that the GR shares desensitization mechanism(s) already present in HEK-293 cells that do not naturally possess the GR.

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