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Expression of Thyrotropin-Releasing Hormone Receptors by Adenovirus-Mediated Gene Transfer Reveals that Thyrotropin-Releasing Hormone Desensitization Is Cell Specific

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SUMMARY

Biological studies of seven-transmembrane region G protein-coupled receptors have been restricted by available techniques for gene transfer into mammalian cells. We have created a highly efficient adenovirus-based expression vector for the thyrotropin-releasing hormone (TRH) receptor (TRH-R), AdCMVmTRHR, to circumvent difficulties encountered when transient or stable plasmid expression systems are used. We show that infection with AdCMVmTRHR results in fully functional TRH-Rs, which can be expressed in a broad range of mammalian cell types, including those resistant to conventional transient transfection. TRH-Rs can be expressed at high levels, up to 2×10^6 receptors/cell. Expression in several cell lines in culture reveals that rapid TRH-R desensitization by TRH and phorbol 12-myristate 13-acetate is cell type specific. The versatility of adenovirus-mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology but also provides a valuable *in vivo* expression vector capable of extending TRH-R studies to animal model systems.

Seven-transmembrane region GPCRs comprise a large family of cell surface regulatory proteins that are now being intensively studied (1). For studies of the molecular details of receptor biology in mammalian cells, expression of wild-type and mutant receptors is usually accomplished by gene transfer, by one of several transfection procedures. Assays using a cell system that permits intracellular replication of the plasmid vector during transient expression studies or establishing transfectants that stably express the receptor of interest allow useful but limited receptor expression. Where transfections yield low levels of receptor expression or in situations where the range of cell types that can be transfected is restricted, this approach has proven to be a limitation to the study of these receptors.

An alternative strategy to plasmid-based receptor expression vectors may be found in the use of adenovirus-mediated gene transfer. Although adenovirus-based vectors for gene expression have been successfully used with a number of mammalian and viral genes (for review, see Ref. 2), they have not, to our knowledge, been used to express any member of the GPCR family, such as the pituitary TRH-R (3–6). A major advantage of using adenovirus-mediated gene transfer is the wide variety of cells that are susceptible to infection by adenovirus, which should permit study of TRH-R biology in a variety of mammalian cell types, including those not amenable to transfection techniques. We used this vector to express TRH-Rs and to study rapid receptor desensitization, an important physiological regulatory process for GPCRs (7, 8), in a number of cell lines in culture. Expression of TRH-Rs on these different cell types allowed us to show that TRH-induced desensitization is cell type specific.

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Experimental Procedures

Materials. Dulbecco's modified Eagle's medium, modified Eagle's medium, Ham's F10 medium, and horse and fetal bovine sera were purchased from GIBCO. Nu-Serum was from Collaborative Research. TRH, methyl-TRH, and PMA were from Sigma. *myo*-[^{3}H]Inositol was from Amersham. [^{3}H]Methyl-TRH was from Du Pont-New England Nuclear. The expression vector pCDM8 was from Invitrogen.

Construction of AdCMVmTRHR. The parent plasmid, pAdCMVmTRHR, was constructed by inserting a 1.2-kilobase *Eco*RI-*Not*I fragment containing the protein-coding region of the mouse TRH-R

ABBREVIATIONS: GPCR, G protein-coupled receptor; TRH, thyrotropin-releasing hormone; TRH-R, thyrotropin-releasing hormone receptor; methyl-TRH, [*N*^{r}-methyl-His]-thyrotropin-releasing hormone; IP, inositol phosphate; PMA, phorbol 12-myristate 13-acetate; HEK, human embryonic kidney; CMV, cytomegalovirus; MOI, multiplicity of infection.

cDNA, nucleotides 233–1462 of plasmid pBSmTRHR (3), into plasmid pGEM2-L3–114 at the *Eco*RI-*Bam*HI site. After digestion with *Eco*RI and use of the Klenow fragment of DNA polymerase I to make blunt DNA ends, *Hind*III linkers were ligated and a 1.4-kilobase *Hind*III fragment containing mouse TRH-R cDNA and the adenovirus E2 poly(A)$^+$ signal sequence was isolated and inserted into the *Hind*III site of the pAdCMV expression cassette, which contains the left end replication and packaging elements of adenovirus, the CMV-1 promoter, and splicing elements from plasmid pML-S.I.S.Cat of Huang and Gorman (9).[1] After verification of the plasmid by restriction site mapping and transient transfection of pAdCMVmTRHR into COS-1 cells to demonstrate TRH-R expression, the virus AdCMVmTRHR was constructed by overlap recombination, as described previously (10).

Briefly, this involves $CaPO_4$ co-transfection of 5 μg of linearized pAdCMV-TRHR (linearized at the *Apa*I site at 13.2 map units) with 1–2 μg of the large fragment of dl309 (3.8–100 map units) onto a 70% confluent 60-mm dish of 293 cells (HEK cells transformed with the E1 region of adenovirus type 5) (11). Successful overlap recombination between the plasmid and large viral fragment (the overlap region between 8.1 map units and 13.1 map units is shared between the plasmid and the large fragment) yields a viable virus due to complementation by the E1 region of 293 cells. The large fragment of adenovirus was made by digesting purified dl309 DNA with *Cla*I (at 2.6 milliunits) and *Xba*I (at 3.8 map units); the DNA was then treated with calf intestine phosphatase to inhibit religation of viral DNA after transfection. Because the 3.8–100-map unit viral fragment lacks the left end origin of replication and the DNA-packaging sequence (present in map unit 0–1), it cannot be replicated or packaged in the absence of recombination. Viral lysates were screened by restriction digestion of viral DNA isolated by a modification of the procedure of Hirt (12). After plaque purification and characterization of individual plaque isolates, the recombinant virus AdCMVmTRHR was grown in 293 cells in suspension. Virus purification (13) from 800 ml of infected 293 cells yielded approximately 6 ml of virus at 2×10^{12} particles/ml (10^{11} plaque-forming units/ml).

Infection with AdCMVmTRHR. Cells were seeded in poly-L-lysine-pretreated wells (3.8 cm^2) and were incubated in medium supplemented with serum, in a humidified atmosphere of 5% CO_2. After a minimum of 4 hr, the medium was aspirated and replaced with 0.3 ml of medium without serum, AdCMVmTRHR (300 particles/cell) was added, and the cells were incubated at 37°. After 1 hr, 0.7–1.0 ml of medium containing serum was added and the incubation was continued for 3–72 hr. Infection with AdCMVmTRHR was performed in an identical manner for all cell types except that the incubation media were different. The media were Dulbecco's modified Eagle's medium supplemented with 5% Nu-Serum for human cervical cancer HeLa cells, monkey kidney COS-1 and CV-1 cells, and rat glioma C6 cells; Ham's F-10 medium with 15% horse serum and 2.5% fetal bovine serum for rat pituitary tumor GHY cells; Dulbecco's modified Eagle's medium with 10% Nu-Serum for mouse pituitary tumor AtT-20 cells and HEK 293 cells; and modified Eagle's medium with 10% fetal bovine serum for human epidermoid KB cells. None of these cell lines express TRH-Rs. Cells were studied 16–24 hr after infection with 300 AdCMVmTRHR particles/cell, which yielded maximal TRH-R expression.

Transfection with pAdCMVmTRHR or pCDM8mTRHR. pCDM8mTRHR is an expression vector in which TRH-R DNA transcription is controlled by a CMV-1 promoter and which contains the simian virus-40 sequence for plasmid replication in COS-1 cells (3). Of two DEAE-dextran methods (14), the one that yielded the higher level of expression was used, depending on the cell type. For HeLa, CV1, and COS-1 cells, a protocol that included incubation with pAdCMVmTRHR or pCDM8mTRHR and DEAE-dextran at 37°, incubation with 0.08 mM chloroquine for 2.5 hr, and addition of dimethylsulfoxide (10%) for 2.5 min was used (3). For GHY, AtT-20, and C6 cells, incubation with plasmid and DEAE-dextran was for 0.5 hr at 4° and no chloroquine or dimethylsulfoxide was added (15). Cells were studied 48–72 hr after transfection, which are times of maximum TRH-R expression.

Meaurement of TRH-R number. Binding of 0.1–7.5 nM [^{3}H]methyl-TRH, an analog of higher affinity and potency than TRH (16), to intact cells was measured as described (17). Binding isotherms were fitted and dissociation constants (K_d values) and receptor numbers (assuming a 1:1 stoichiometry of methyl-TRH and receptor) were obtained with the INPLOT program (GraphPAD). In some experiments, receptor number was calculated using the following equation: fractional occupancy = $1/[1 + (K_d/L)]$. Where receptor number is given as sites per cell, it is assumed that all cells in the population express equal numbers of TRH-Rs. This appears to be the case with infections using 300 AdCMVmTRHR particles/cell.[2]

Measurement of TRH response. Infected or transfected cells were labeled for 24 hr with *myo*-[^{3}H]inositol and stimulated with TRH or methyl-TRH in a balanced salt solution containing 10 mM LiCl, and [^{3}H]IPs were measured as described (18).

Measurement of desensitization and inhibition by PMA. Cells were incubated in medium containing serum and *myo*-[^{3}H]inositol (1 μCi/ml) for 24 hr before infection and were studied 16–24 hr after infection. The desensitization protocol was as described (19), except that all incubations were at 37°. Stimulation by TRH was with cells incubated in medium containing serum and *myo*-[^{3}H]inositol, to prevent depletion of ^{3}H-labeled phosphoinositide substrate. The rate of IP formation was determined by linear regression analysis of the amount of [^{3}H]IPs, expressed as percentage of ^{3}H-labeled phosphoinositides, measured at 3 times during a 30-min incubation. The desensitized rate was measured after 60 min of stimulation with 1 μM TRH, by addition of LiCl to a final concentration of 10 mM. The initial rate of TRH-stimulated IP formation was measured by adding TRH and LiCl simultaneously (at 60 min, in parallel with the desensitized cells). In experiments with PMA, PMA was dissolved in dimethylsulfoxide and was added 60 min before TRH and LiCl, to a final concentration of 0.1 μM.

Results and Discussion

We constructed a highly efficient, replication-defective, adenovirus vector, AdCMVmTRHR, that contains the coding sequence of the mouse TRH-R under the control of the CMV-1 promoter and RNA-processing elements inserted at the E1 region of a parent adenovirus, dl309 (20). The strategy used for the construction of AdCMVmTRHR involved replacement of the adenovirus E1A and E1B genes to create a replication-defective TRH-R-expressing virus. The first step in construction was to create the appropriate adenovirus vector plasmid containing the mouse TRH-R cDNA (Fig. 1). This plasmid was then used in a co-transfection with a restriction fragment of adenovirus that lacks the left end origin of replication and the adenovirus packaging sequence. After homologous recombination between the plasmid and the viral fragment in the E1A- and E1B-complementing 293 cell line, the AdCMVmTRHR virus was produced.

One of the virtues of gene expression by virus infection is the ability to deliver, in a dose-dependent manner, a predetermined copy number of viral DNA templates into cell types that express the receptor for adenovirus type 5. AdCMVmTRHR infection of GHY cells (a subclone of pituitary tumor cells that does not express TRH-Rs but is derived from the most well

[1] M. Alvira and E. Falck-Pedersen. A new multifunctional adenovirus expression vector useful for overexpression of a variety of gene products. Manuscript in preparation.

[2] M. C. Gershengorn, M. Heinflink, D. R. Nussenzveig, P. M. Hinkle, and E. Falck-Pedersen, unpublished observations.

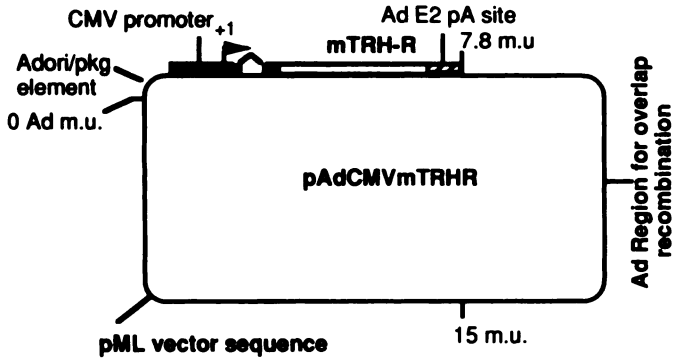

Fig. 1. Schematic diagram of the parent plasmid pAdCMVmTRHR, which was used for the construction of the recombinant virus AdCMVmTRHR. The left end of adenovirus (*Ad*) starts at position 1. Adenovirus sequence from nucleotide 1 to nucleotide 353 contains the origin of replication and the viral packaging sequence. Adenovirus sequence from nucleotide 354 to nucleotide 2800 was deleted and replaced with the CMV-1 promoter, splice elements, the protein-coding region of the mouse TRH-R cDNA sequence, and the E2 poly(A)$^+$ (*pA*) site. The left end adenovirus sequence from nucleotide 2800 to nucleotide 5776 serves as the region for homologous recombination.

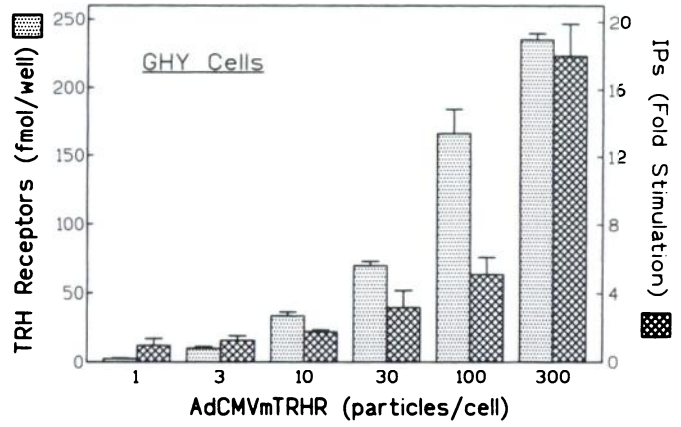

Fig. 2. Expression of TRH-Rs and stimulation of IP formation in GHY cells infected with AdCMVmTRHR. GHY cells were infected with AdCMVmTRHR at the indicated MOIs. After 24 hr, the expression of TRH-Rs and TRH stimulation of IP formation in cells labeled with *myo*-[^{3}H] inositol were measured as described in Experimental Procedures. *Bars*, mean ± standard deviation of triplicate determinations in a representative experiment.

studied cell model of TRH-R biology) (21) provides a means to control the level of TRH-R expression over a wide range, in a manner dependent on virus input (Fig. 2). The binding affinity of TRH-Rs produced by viral infection was the same as reported previously (data not shown) (3). There was a progressive increase in the level of TRH-R expression up to 240 fmol/well with infection by 300 AdCMVmTRHR particles/cell. This corresponds to approximately 1×10^6 receptors/GHY cell, assuming that all cells express equal numbers of TRH-Rs. This compares with levels of $50–200 \times 10^3$ endogenous TRH-Rs/GH cell (22, 23) and $30–60 \times 10^3$ TRH-Rs/cell in stably transfected GH cell lines (15). Expression was evident after 5 hr, the earliest time studied, and attained a peak level after 24 hr that was constant through the end of the experiment at 72 hr (data not shown). There was no indication of altered cell function due to virus infection at the MOI used in this study. We noticed at higher MOI (3000 particles/cell) that high receptor numbers were detected at all time points, but there was evidence of a cytotoxic effect on the GHY cells after 24 hr, presumably due to cytopathic effects known to occur with adenovirus at extremely high doses (data not shown).

A proximal step after TRH-R activation is stimulation of the formation of IP second messengers (24, 25). We measured TRH stimulation of IP formation in GHY cells after infection with AdCMVmTRHR to demonstrate that receptor expressed from the virus was in fact fully functional. It was shown previously for endogenous TRH-Rs expressed in GH cell lines that the magnitude of IP second messenger formation was proportional to receptor number (22, 23, 26, 27). Using IP second messenger formation as a measure of receptor function, we found that the receptor number and the magnitude of IP second messenger formation stimulated by TRH were proportional to the dose of AdCMVmTRHR input (Fig. 2). Thus, AdCMVmTRHR-infected GHY cells express TRH-Rs that exhibit normal binding and coupling.

In addition to controlled levels of receptor expression, the general usefulness of AdCMVmTRHR as a vector for expression of TRH-Rs and its advantage over transfection are illustrated in Fig. 3. Expression of TRH-Rs after infection with AdCMVmTRHR is directly compared with expression of TRH-Rs produced after transfection of the same cell type with pAdCMVmTRHR, the plasmid used for virus construction. We compared expression in rat pituitary tumor GHY cells, mouse pituitary tumor AtT-20 cells, human cervical cancer HeLa cells,

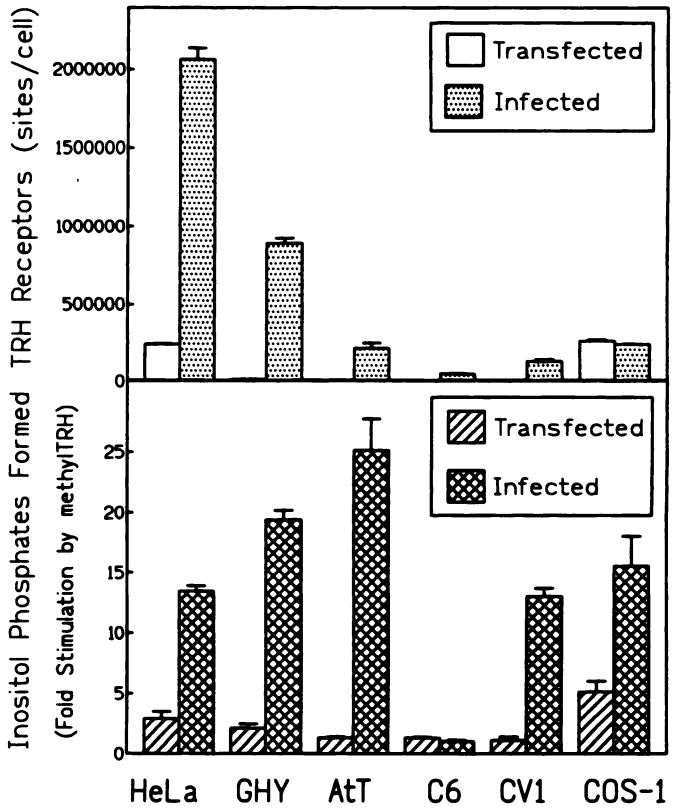

Fig. 3. Comparison of effects of infection with AdCMVmTRHR and transfection with pAdCMVmTRHR on expression of TRH-Rs and methyl-TRH responsiveness in six mammalian cell lines. The levels of TRH-R expression (*upper*) and methyl-TRH stimulation of [^{3}H]IP formation (*lower*) were measured as described in Experimental Procedures. *Upper*, data are presented as number of receptors per cell, assuming that all cells express equal numbers of TRH-Rs. *Bars in both panels*, mean ± standard deviation of triplicate determinations in a representative experiment that was performed three times.

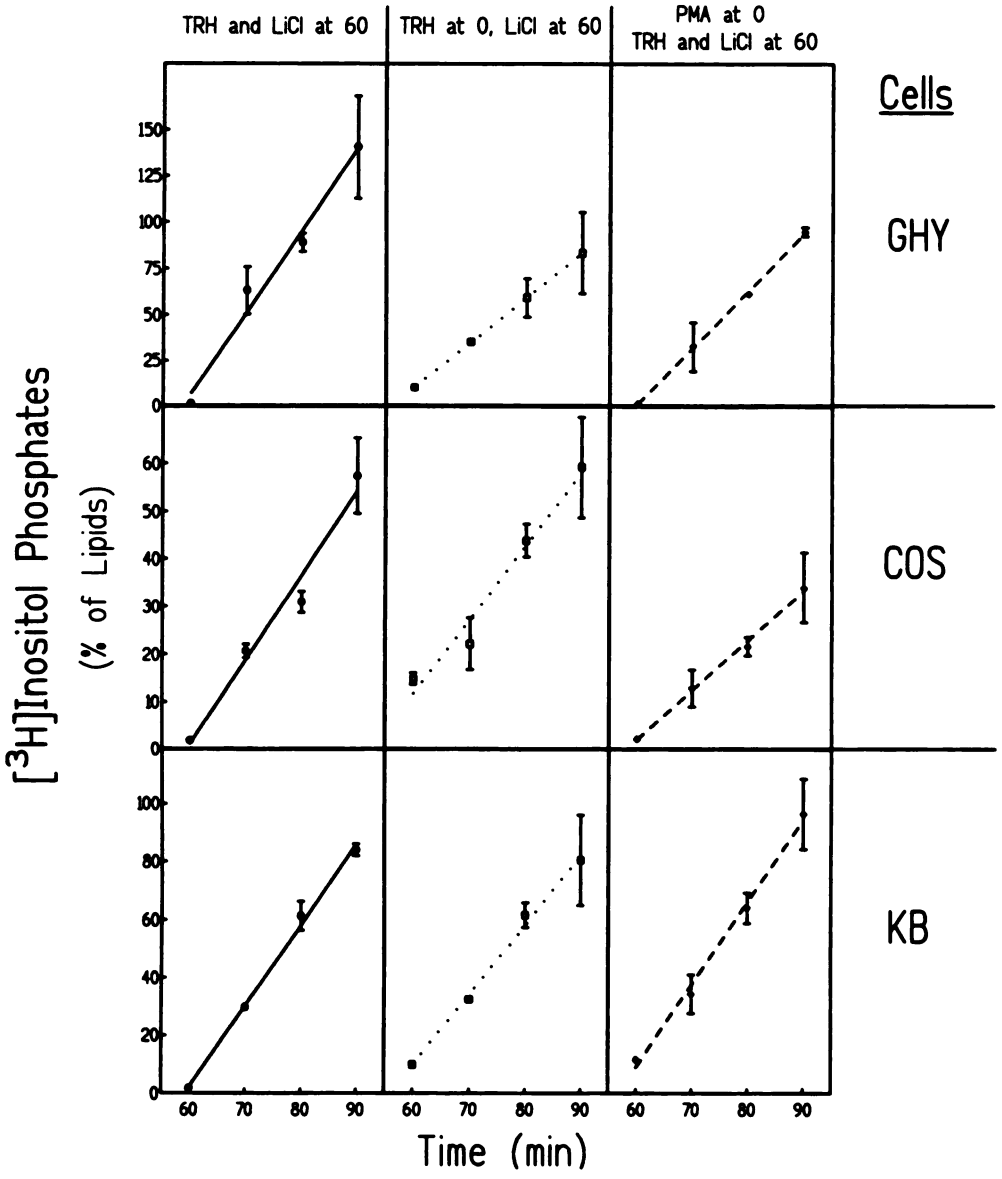

Fig. 4. TRH-induced desensitization and PMA-induced inhibition of the TRH response in AdCMVmTRHR-infected GHY, COS-1, and KB cells. GHY, COS-1 (COS), and KB cells were infected with 300 AdCMVmTRHR particles/cell, and TRH-induced desensitization and PMA-induced inhibition of the TRH response were measured as described in Experimental Procedures. The data represent the mean ± standard deviation of triplicate determinations in a representative experiment that was performed two or three times.

TABLE 1

TRH-induced desensitization and PMA-induced inhibition of the TRH response in AdCMVmTRHR-infected cells

The data represent the mean ± standard deviation of triplicate determinations in two or three experiments.

Cell line	TRH-induced desensitization	PMA-induced inhibition
	%	%
GHY	49 ± 5.2	25 ± 4.6
AtT-20	41 ± 4.7	37 ± 4.0
COS-1	12 ± 11	37 ± 8.0
HeLa	15 ± 15	24 ± 4.0
HEK 293	0 ± 1	95 ± 1.0
KB	5.5 ± 11	0 ± 6.0

human epidermoid KB cells, HEK 293 cells, rat glioma C6 cells, and monkey kidney CV1 and COS-1 cells. These cell lines were chosen because they represent a wide variety of cell types that do not express TRH-Rs. HeLa cells were studied because they are readily infected with adenovirus. GHY cells were studied because they are a subclone of the cells in which endogenous TRH-Rs have been most well studied. COS-1 cells were studied because they are a commonly used, transformed cell line that permits high levels of expression during transient assays. TRH-Rs expressed on the surface of these cells after infection with AdCMVmTRHR bound methyl-TRH with the same affinity as did native TRH-Rs on mouse pituitary cells (17) or TRH-Rs stably (15) or transiently (3, 28) expressed on several different cell types, including COS-1 and HeLa cells, after transfection. The dissociation constant for methyl-TRH binding was 1.09 ± 0.26 nM (data not shown).

The most interesting and important finding from this experiment was that there was a higher level of TRH-R expression in every cell type except COS-1 cells when gene transfer was mediated by AdCMVmTRHR infection, compared with transfection with pAdCMVmTRHR (Fig. 3, *upper*) or with a plasmid, pCDM8mTRHR (3), that can replicate efficiently in COS-1 cells (data not shown). Under the conditions studied, there were marked differences among the various cell types in the levels of expression of TRH-Rs after infection by AdCMVmTRHR. Although we have not systematically determined the optimal conditions for AdCMVmTRHR-mediated TRH-R expression in each cell type, we think that these differences may be related to intrinsic characteristics of the different cell types, rather than differences in conditions needed for optimal infection. For example, there may be cell-specific differences in efficiencies of adenovirus infection, perhaps related to the number of adenovirus receptors, in expression of exogenous genes in general or of TRH-R specifically, or in turnover of TRH-Rs. Thus, infection by AdCMVmTRHR led to higher levels of TRH-R expression in a wider range of cell types than did transient transfection.

We measured methyl-TRH stimulation of IP formation in all cell types after infection by AdCMVmTRHR (Fig. 3, *lower*). Uninfected GHY, AtT, HeLa, KB, HEK 293, C6, CV1, and COS-1 cells did not respond to methyl-TRH. In parallel with the number of TRH-Rs, there was a greater stimulation of IP formation by methyl-TRH in all cell types after infection by AdCMVmTRHR than after transfection. In contrast to the results seen in Fig. 2 for varied receptor number in a single cell type, when different cell types that expressed different receptor numbers (at a fixed concentration of virus) were compared there was no correlation between the magnitude of IP formation stimulated by methyl-TRH and the number of TRH-Rs. For example, methyl-TRH stimulation of IP formation was greater in AtT-20 cells, which expressed TRH-Rs at a lower number, than in HeLa cells, with a greater number of TRH-Rs. One explanation for this observation may be that there are differences in post-receptor components of the signal transduction cascades within these different cell types. These experiments also revealed that the magnitude of response to methyl-TRH in COS-1 cells was greater after infection than after transfection, even though the total numbers of receptors were similar. We think this may be because all COS-1 cells expressed TRH-Rs after AdCMVmTRHR infection, whereas only a fraction of the transfected cells expressed TRH-Rs.

In rat GH_3 pituitary cells naturally expressing TRH-Rs, TRH stimulation of IP formation is rapidly desensitized (19). This effect occurs before any decrease in the number of TRH-Rs ("down-regulation") (17, 29). The TRH response is also blunted when GH_3 cells are preincubated with phorbol esters, which are activators of protein kinase C (30). Although the molecular mechanisms of TRH-induced desensitization and of PMA-induced inhibition of the TRH response have not been elucidated, it is likely that they are mediated by receptor phosphorylation (31). Because different cell types contain different complements of protein kinases, it is possible that TRH-induced desensitization and PMA-induced inhibition of the TRH response are cell type specific. We used AdCMVmTRHR infection to express TRH-Rs in several different cell types (Fig. 4; Table 1) and found that TRH-induced desensitization and PMA inhibition of the TRH response are cell specific. TRH-induced desensitization and PMA-induced inhibition of the TRH response were observed in both pituitary cell types, i.e., AdCMVmTRHR-infected GHY cells and AdCMVmTRHR-infected AtT-20 cells. These effects are indistinguishable from those measured with endogenous TRH-Rs in GH_3 cells (19). In contrast, in AdCMVmTRHR-infected COS-1 cells, AdCMVmTRHR-infected HeLa cells, and AdCMVmTRHR-infected HEK 293 cells the response to TRH did not desensitize, whereas PMA inhibited the TRH response. In AdCMVmTRHR-infected KB cells, which expressed $1.16 \pm 0.02 \times 10^6$ TRH-Rs/cell, there was no TRH-induced desensitization and PMA did not inhibit the TRH response. Thus, in this limited survey of cell lines, TRH-induced desensitization and PMA-induced inhibition of the TRH response were found only in two rodent pituitary-derived cell types. This does not mean that TRH-induced desensitization will be found only in pituitary-derived cells. PMA-induced inhibition of TRH responsiveness but not TRH-induced desensitization was observed in monkey kidney-derived cells, human cervical cancer cells, and HEK cells. Neither TRH-induced desensitization nor PMA-induced inhibition of TRH responsiveness was found in human epidermoid-derived cells. These findings indicate that TRH-induced desensitization is not mediated primarily by protein kinase C.

A number of aspects of GPCR biology may vary when receptors are expressed in different cell types. For example, the same GPCR may activate different signal transduction pathways when expressed in different cell types (32). Agonist-induced desensitization, which is a process that commonly accompanies activation of GPCRs, appears to be mediated by a conserved set of intracellular regulatory proteins, including protein kinases and arrestin-like proteins (33, 34). Our data demonstrate that desensitization of TRH-Rs may occur in some cell types (GHY and AtT-20 cells) but not in others (COS-1, HeLa, HEK 293, and KB cells). Although we have found TRH-induced desensitization only in cell lines derived from the pituitary gland, we cannot conclude that TRH-R desensitization occurs only in pituitary-derived cells, because we have studied a limited number of cell lines.

In summary, we have created a replication-defective adenovirus, AdCMVmTRHR, that was constructed for high efficiency expression of TRH-Rs. Using this virus, we have been able to express TRH-Rs at high levels in a variety of mammalian cell types and to study several aspects of TRH-R biology in different cell environments. We found that desensitization of the TRH response is cell type specific, occurring only in pituitary-derived cells in a limited survey of cell types. This finding that an aspect of TRH-R biology is different in different cell types is important, because TRH-Rs are normally expressed on several cell types (35).

We conclude that adenovirus-mediated gene transfer is an excellent method for expression of TRH-Rs, and we suggest that this approach could be extended for expression of other cell receptors in many cell types. The versatility of adenovirus-mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology but also should provide a valuable *in vivo* expression vector capable of extending TRH-R studies to animal model systems.

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