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A Disulfide Bond between Conserved Extracellular Cysteines in the Thyrotropin-releasing Hormone Receptor Is Critical for Binding*

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The assumption that a disulfide bond is present between two highly conserved cysteines in the extracellular loops of G protein-coupled receptors and is critical for receptor function has been cast in doubt. We undertook to determine whether a disulfide bond important for binding or activation is present in the thyrotropin-releasing hormone (TRH) receptor (TRH-R). Studies were performed with cells expressing wild-type (WT) and mutant receptors in the absence or presence of the reducing agent dithiothreitol (DTT). The affinity of WT TRH-R was 16–22-fold lower in the presence of DTT than in the absence of DTT. Mutant receptors were constructed in which Ala was substituted for conserved Cys-98 and Cys-179 of extracellular loops 1 and 2, respectively, and for the nonconserved Cys-100. C98A and C179A TRH-Rs did not exhibit high affinity binding. These mutant receptors were capable of stimulating inositol phosphate second messenger formation to the same extent as WT TRH-Rs but with a markedly lower potency. The affinities of C98A and C179A TRH-Rs, estimated from their potencies, were 4400- and 640-fold lower, respectively, than WT TRH-R. The estimated affinities of neither C98A nor C179A TRH-R were decreased by DTT. In contrast, the estimated affinity of C100A TRH-R was not different from WT TRH-R and was DTT sensitive. Moreover, the effect of mutating both Cys-98 and Cys-179 was not additive with the effects of the individual mutations. These data provide strong evidence that Cys-98 and Cys-179 form a disulfide bond. This interaction is not involved in receptor activation but is critical for maintaining the high affinity conformation of TRH-R.

Seven transmembrane-spanning, guanine nucleotide-binding (G) protein-coupled receptors (GPCRs)[1] constitute a large family of cell surface regulatory molecules (1, 2). With rare exceptions (for example, the Mas oncogene and cannabinoid receptors (3)), members of this family contain a cysteine in the putative first extracellular loop (EC-1) near the top of the third transmembrane domain (TM-3) and another cysteine in EC-2. It has been assumed that this pair of Cys residues forms a disulfide bond in most GPCRs (2). This linkage has been proposed to be important to allow the receptor to attain a normal conformation during synthesis, for normal expression on the cell surface or to maintain normal function, in particular, normal binding and activation. Data consistent with the presence of a disulfide bond between these two conserved Cys residues have been reported for some receptors of the GPCR family, but there is evidence that these residues do not always participate in this linkage. Evidence in support of this disulfide linkage has been primarily of two types. First, it has been shown that the binding affinity of a number of GPCRs is decreased under reducing conditions. This effect, however, may involve reductions of disulfides other than this proposed bond. And second, substitution of one or the other of these Cys residues has led to decreased binding affinity, expression, or activation. These effects may be due to loss of the disulfide bond or could be caused by an adverse effect of the substituting amino acid (see below).

Rhodopsin, β_2-adrenergic receptors, and muscarinic acetylcholine receptors are the best studied GPCRs with regard to the presence of an extracellular disulfide bond. In experiments with mutants of rhodopsin in which one or both of the cysteines were substituted by alanine (4), findings were consistent with the presence of a disulfide bond between the conserved Cys residues. In contrast to previous studies in which the Cys residues were substituted with Ser (see below) (5–7), these Cys to Ala mutants of rhodopsin were expressed normally and bound retinal normally; Cys to Ala mutants did exhibit a defect in the stability of an activated intermediate, metarhodopsin II. In the β_2-adrenergic receptor, there may be two extracellular disulfide bonds. Single substitution with Val of all four Cys residues in the β_2-adrenergic receptor caused decreases in binding affinity and receptor expression, but the residues linked by disulfide bonds were not identified (8, 9). In fact, data from a series of β_2-adrenergic receptor mutants in which the cysteines were substituted with Ala show there is no disulfide bond between the two conserved cysteines (10). The data from experiments in which muscarinic acetylcholine receptors have been mutated are more difficult to interpret because the conserved Cys residues were substituted by Ser (11) (see below). (Findings from experiments in which muscarinic receptors were labeled with a disulfide-specific reagent are consistent with the presence of a disulfide bond between the conserved cysteines (12, 13).) The data for other members of the GPCR family are also consistent with the presence of this bond but are not conclusive. For example, it has been reported that single substitution of any of four extracellular cysteines with glycine in the angiotensin II AT_1 receptor leads to a 10-fold decrease in affinity (14).

A number of GPCRs have been studied by mutation of the conserved cysteines to serines. The data from these experiments are difficult to interpret because the hydrophilic nature

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[1] The abbreviations used are: GPCR, guanine nucleotide-binding protein-coupled receptor; TRH, thyrotropin-releasing hormone; TRH-R, TRH receptor; TM, transmembrane domain, *e.g.* TM-3, third TM; EC, extracellular domain, *e.g.* EC-1, first extracellular loop; DTT, dithiothreitol; K_i, equilibrium inhibitory constant; IP, inositol phosphate; MeTRH, [N^{τ}-*methyl*-His]TRH; EC_{50}, half-maximally effective concentration; WT, wild type.

of the hydroxyl group of serine may have adversely affected receptor conformation. This idea is based on findings with rhodopsin. It was observed that substitution of the conserved Cys residues with Ser in rhodopsin resulted in more marked deleterious effects than substitution with Ala (4) in that Cys to Ser mutants exhibited abnormal levels of expression and glycosylation and an inability to bind retinal (5–7). Thus, studies of, for example, muscarinic acetylcholine (11) and thyroid-stimulating hormone receptors (15, 16) in which mutant receptors with Ser substitutions demonstrated loss of binding must be interpreted cautiously.

Thus, it cannot be concluded that conserved Cys residues in EC-1 and EC-2 form a disulfide bond in all GPCRs. Moreover, if a disulfide bond is present in a GPCR it is not necessarily critical for attainment of the native conformation, level of expression, or function of the receptor.

The TRH receptor (TRH-R) is a member of the GPCR family (17). In previous studies, binding of TRH has been found to decrease under disulfide bond reducing conditions (18, 19). The studies presented here were undertaken to determine whether the conserved Cys at position 98 (Cys-98) of EC-1 and the conserved Cys-179 of EC-2 of TRH-R are covalently linked by a disulfide bond and whether this linkage is necessary for normal TRH-R function. Our data, based on single and double mutations of these Cys residues and on the effects of the reducing agent dithiothreitol (DTT) on these mutants, offer strong evidence that a disulfide bond is present between Cys-98 and Cys-179 and, furthermore, that this linkage is critical for maintaining the high affinity conformation of TRH-R.

EXPERIMENTAL PROCEDURES

Materials—TRH was purchased from Calbiochem, MeTRH and DTT from Sigma, and [^{3}H]MeTRH from DuPont NEN. The expression vector pCDM8 was from Invitrogen. Dulbecco's modified Eagle's medium and fetal calf serum were from Life Technologies, Inc. Nu-Serum was from Collaborative Research. Restriction endonucleases were from New England Biolabs.

Mutagenesis—The full-length mouse TRH-R cDNA in pCDM8 (pCDM8mTRHR) (20) was used for mutation and transformation. Mutants were prepared by polymerase chain reaction, and plasmid sequences were confirmed by the dideoxy chain termination method. C98A and C179A TRH-Rs were digested with *Bsp*HI and *Nsi*1 and the 184-base pair restriction fragment from C98A TRH-R subcloned into C179A TRH-R to construct C98A/C179A TRH-R. The double mutant C98S/C179S TRH-R was prepared by polymerase chain reaction using C98S TRH-R as a template.

Cell Culture and Transfection—COS-1 cells and AtT-20 cells were maintained and transfected as described (21, 22).

Inositol Phosphate (IP) Formation—Assays were performed as described (21). To study the effects of reduction of disulfide bonds on IP formation, cells expressing TRH-Rs were preincubated for 1 h at 37 °C in the absence or presence of 10 mM DTT. LiCl (10 mM) and various concentrations of TRH were then added for an additional 1 h at 37 °C.

Receptor Binding Studies—Assays were performed as described (21). To study the effects of reduction of disulfide bonds on binding, cells expressing TRH-Rs were preincubated for 1 h at 37 °C in the absence or presence of 10 mM DTT. [^{3}H]MeTRH (1 nM, in the absence of DTT, or 5–10 nM, in the presence of DTT) and various concentrations of unlabeled MeTRH were then added for an additional 1 h at 37 °C.

Statistical Analyses—Curves were fitted by nonlinear regression and drawn with GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

To begin to assess the importance of disulfide bonds in TRH-R for binding, the effect of the reducing agent DTT was studied. WT TRH-Rs were stably expressed in AtT-20 pituitary cells (23, 24), and binding affinities for MeTRH were measured in the absence or presence of DTT (Fig. 1*A*). In the absence of DTT, the K_i for MeTRH was 1.3 nM, and the affinity was lowered 16-fold in the presence of DTT. For WT TRH-Rs transiently expressed in COS-1 cells, the K_i for MeTRH was 2.4 nM

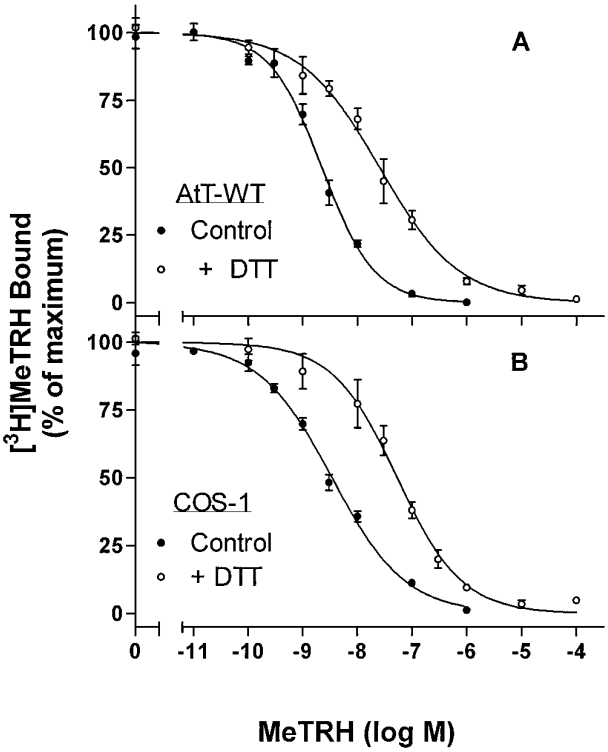

FIG. 1. **Effect of DTT on binding to WT TRH-Rs.** Competition binding for [^{3}H]MeTRH by unlabeled MeTRH in the absence or presence of DTT as described under "Experimental Procedures" is shown. WT TRH-Rs were expressed stably in AtT-20 cells (*A*) or transiently in COS-1 cells (*B*). Duplicate determinations were obtained in two to four experiments.

in the absence of DTT and was lowered 22-fold in the presence of DTT (Fig. 1*B*, Table I). These data, from two different cell lines, indicate the importance of a reducible disulfide bond(s) in TRH-R for high affinity binding.

To determine which cysteines were contributing to this effect, the conserved Cys-98 and Cys-179, as well as the nonconserved Cys-100, were mutated to alanine, expressed transiently in COS-1 cells, and assayed for binding (Table I). The affinity of C100A TRH-R for MeTRH was the same as WT TRH-R. In contrast, C98A and C179A TRH-Rs showed no specific binding with up to 10 nM [^{3}H]MeTRH. To estimate the affinity of these presumably low affinity receptors, the EC_{50} values of TRH for stimulation of IP formation were measured. The maximal extents of stimulation of WT, C98A, C100A, and C179A TRH-Rs were the same, which is consistent with the idea that the efficacies of these receptors are similar. Therefore, relative potencies were used to estimate relative affinities (25). The EC_{50} of TRH in cells expressing WT TRH-R was 0.84 nM. The EC_{50} values of C98A, C100A, and C179A TRH-Rs were 4400-, 2-, and 640-fold higher than that of WT TRH-R (Fig. 2*A*, Table I). This indicated that C98A and C179A TRH-Rs are expressed on the cell surface and that Cys-98 and Cys-179 are critical for high affinity binding. In contrast, Cys-100 does not appear to be important for high affinity binding. To determine whether there was an interaction between Cys-98 and Cys-179, the double mutant C98A/C179A TRH-R was expressed in COS-1 cells and the EC_{50} of TRH was measured. If mutation of Cys-98 and Cys-179 were affecting the same bond, one would expect the effect of the double mutation to be similar to the effects of the individual mutations. This is what was found. The EC_{50} for C98A/C179A TRH-R was the same as that for C98A TRH-R, indicating that mutation of either Cys-98 or Cys-179 resulted in loss of the same bond.

TABLE I
Binding and activation of WT and mutant TRH-Rs expressed in COS cells in the absence or presence of DTT

Values are expressed as means (95% confidence intervals) based on duplicate determinations in two to eleven experiments. ND, not done.

	Binding of WT and mutant TRH-Rs (K_i, nM)	
	WT	C100A
−DTT	2.4 (1.4–4.0)	2.1 (1.1–3.7)
+DTT	53 (30–91)	ND

	Activation of WT and mutant TRH-Rs (EC_{50}, nM)				
	WT	C98A	C100A	C179A	C98A/C179A
−DTT	0.84 (0.67–1.0)	3,700 (2,900–4,700)	1.8 (1.5–2.1)	540 (410–700)	4,700 (3,500–6,400)
+DTT	10 (5.1–20)	1,100 (770–1,500)	16 (12–22)	330 (180–610)	ND

	C98S	C179S	C98S/C179S
−DTT	170,000 (122,000–243,000)	18,000 (8,600–36,000)	360,000 (240,000–520,000)

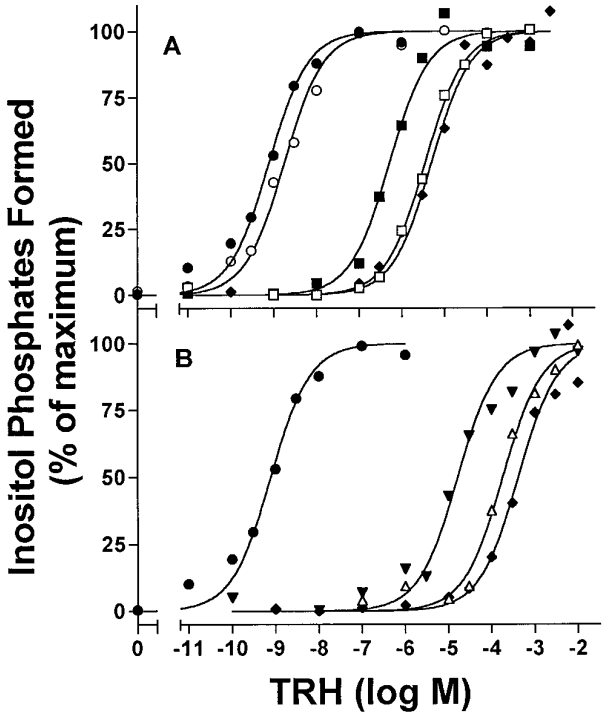

FIG. 2. **Stimulation of inositol phosphate formation by TRH in COS-1 cells expressing WT or mutant TRH-Rs.** Cysteines were mutated to alanine (*A*) or serine (*B*). Duplicate determinations were obtained in two to eleven experiments. *A:* ●, WT; ○, C100A; ■, C179A; □, C98A; ♦, C98A/C179A. *B:* ●, WT; ▼, C179S; △, C98S; ♦, C98S/C179S.

To confirm these findings, Cys-98 and Cys-179 were mutated to Ser. The maximal stimulations by TRH in COS-1 cells expressing C98S, C179S, and C98S/C179S TRH-Rs were 64 ± 9, 53 ± 10, and 97 ± 5% of that in cells expressing WT-TRH-Rs. The EC_{50} values for TRH in cells expressing C98S, C179S, and C98S/C179S TRH-Rs were 200,000-, 21,000-, and 430,000-fold higher than that of WT TRH-R (Fig. 2*B*, Table I). Therefore, as for the mutations to Ala, single mutations of Cys-98 or Cys-179 to Ser resulted in loss of high affinity binding. The results with the doubly mutated receptor were consistent with the presence of a disulfide bond between Cys-98 and Cys-179 because the estimated affinity of C98S/C179S TRH-R was similar to C98S TRH-R and was much less than the additive losses of C98S and C179S TRH-Rs that would be expected if these mutations affected different aspects of TRH-R. The recovery of a WT level of maximal stimulation by the double Ser mutant is consistent with an interaction between Cys-98 and Cys-179 also. That is, the two Ser residues in C98S/C179S TRH-R may form a hydrogen bond that could function in place of the native disulfide bond and restore efficacy to the double mutant receptor.

To obtain additional evidence that a disulfide bond was present between Cys-98 and Cys-179, we measured the effects of DTT on the EC_{50} values of TRH for stimulation of IPs in cells expressing mutant receptors. In AtT-20 cells expressing WT TRH-Rs, the EC_{50} in the presence of DTT was 120-fold higher than in the absence of DTT (0.66 (0.42−1.0) nM *versus* 78 (53−120) nM) demonstrating that effects of DTT on affinity could be assessed through measurement of IP formation. The effects of DTT on estimated affinities of WT and mutant receptors expressed in COS-1 cells were then measured (Table I). In the presence of DTT, the EC_{50} values of WT and C100A TRH-Rs were 12- and 9.3-fold higher, respectively, compared with stimulation in the absence of DTT, indicating the presence of a disulfide bond important for binding. In contrast, the EC_{50} values of C98A and C179A TRH-Rs were lower, 30 and 61%, respectively, in the presence of DTT than in the absence of DTT. This indicates that the critical disulfide bond present in WT TRH-R is absent in C98A and C179A TRH-R. This is consistent with there being a disulfide link specifically between Cys-98 and Cys-179 in WT TRH-R that is important for high affinity binding.

DISCUSSION

We conclude there is a disulfide bond between the conserved Cys-98 of EC-1 and the conserved Cys-179 of EC-2 in TRH-R and that this bond is necessary to constrain TRH-R in a high affinity conformation. These conclusions are based on the following observations: 1) high affinity TRH binding is decreased by DTT in WT TRH-Rs; 2) substitution of either of the conserved Cys residues decreased estimated TRH binding affinity whereas substitution of nearby Cys-100 did not affect binding affinity; 3) estimated binding affinity was not decreased by DTT in mutants in which Cys-98 or Cys-179 were substituted by Ala; 4) substitution of both Cys residues by Ala did not decrease estimated affinity more than substitution of Cys-98 alone; and 5) the double mutant C98S/C179S TRH-R, in which both cysteines were substituted with serines, was capable of stimulating a higher level of IP formation than C98S or C179S TRH-Rs. This last point is consistent with the idea that single Ser substitutions are more deleterious to receptor expression or efficacy than the double mutant because in the double mutant the two Ser residues may form a hydrogen bond that could simulate the function of the lost disulfide bond. Similarly, single substitutions of conserved Cys residues with Ser in the parathyroid hormone/parathyroid hormone-related peptide receptor were found to cause greater decreases in expression of this receptor than that observed with the double mutant (26).

In contrast to the effect on WT TRH-Rs, pretreatment with the reducing agent DTT did not lower the potencies of C98A or C179A TRH-Rs but increased them. This suggests that DTT affects another disulfide bond(s) in TRH-R or in another cellular protein(s) in addition to the disulfide link between Cys-98 and Cys-179. As reduction of this disulfide bond(s) increases the apparent potency of TRH, the effect of breaking the Cys-98/Cys-179 disulfide bond is underestimated. This would explain, in part, the greater decrease in estimated affinity, which is based on relative potencies, noted with Cys-98 or Cys-179 mutants than that observed upon incubating WT TRH-R with DTT. Other factors that may account for these differences may be the more deleterious effects of either a methyl group (in Ala)

or a hydroxyl group (in Ser) rather than free sulfhydryl groups (in 2 Cys) and differences between preventing formation of a disulfide bond during synthesis and folding of the receptor (in the substituted mutants) compared with reduction of a disulfide bond in a correctly folded WT TRH-R after its insertion into the plasma membrane.

Several different roles have been ascribed to the putative disulfide bond between EC loops of GPCRs. It is possible that this disulfide linkage may form during the early stages of receptor synthesis and be necessary for the normal folding and insertion into the membrane of these cell surface proteins. There is, however, no direct data to support this idea and, in particular, the timing of formation of this disulfide bond during synthesis has not been determined in any GPCR. In rhodopsin, the EC disulfide bond appears not to be required for binding the ligand retinal but for the stability of an activated intermediate, metarhodopsin II (4). In TRH-R (this paper), the EC disulfide between these conserved Cys residues is necessary to constrain the receptor in a conformation that can attain high affinity binding. By contrast, two other EC disulfide linkages may be used to maintain a high affinity form of β_2-adrenergic receptors (10). The conserved EC disulfide bond, however, is not required for full apparent efficacy in GPCRs.

In conclusion, we have provided strong evidence for a disulfide bond between the conserved Cys-98 of EC-1 and the conserved Cys-179 of EC-2 in TRH-R and have demonstrated the critical importance of this disulfide link for high affinity binding of TRH. Based on our previous studies showing the binding pocket for TRH to be within the TM bundle (27), we propose that the extracellular disulfide link is essential for constraining the TM helices of TRH-R in the proper binding conformation.

REFERENCES

1. Baldwin, J. M. (1994) *Curr. Opin. Cell Biol.* **6,** 180–190
2. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. F. (1994) *Annu. Rev. Biochem.* **63,** 101–132
3. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Sealfon, S. C. (1992) *DNA Cell Biol.* **11,** 1–20
4. Davidson, F. F., Loewen, P. C., and Khorana, H. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4029–4033
5. Karnik, S. S., Sakmar, T. P., Chen, H.-B., and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8459–8463
6. Doi, T., Molday, R. S., and Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4991–4995
7. Karnik, S. S., and Khorana, H. G. (1990) *J. Biol. Chem.* **265,** 17520–17524
8. Dixon, R. A. F., Sigal, I. S., Candelore, M. R., Register, R. B., Scattergood, W., Rands, E., and Strader, C. D. (1987) *EMBO J.* **6,** 3269–3275
9. Dohlman, H. G., Caron, M. G., Deblasi, A., Frielle, T., and Lefkowitz, R. J. (1990) *Biochemistry* **29,** 2335–2342
10. Noda, K., Saad, Y., Graham, R. M., and Karnik, S. S. (1994) *J. Biol. Chem.* **269,** 6743–6752
11. Savarese, T. M., Wang, C.-D., and Fraser, C. M. (1992) *J. Biol. Chem.* **267,** 11439–11448
12. Kurtenbach, E., Pedder, E. K., Curtis, C. A. M., and Hulme, E. C. (1990) *Biochem. Soc. Trans.* **18,** 442–443
13. Kurtenbach, E., Curtis, C. A. M., Pedder, E. K., Aitken, A., Harris, A. C. M., and Hulme, E. C. (1990) *J. Biol. Chem.* **265,** 13702–13708
14. Yamano, Y., Ohyama, K., Chaki, S., Guo, D.-F., and Inagami, T. (1992) *Biochem. Biophys. Res. Commun.* **187,** 1426–1431
15. Kosugi, S., Ban, T., Akamizu, T., and Kohn, L. D. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1754–1762
16. Gustavsson, B., Westermark, B., and Heldin, N.-E. (1994) *Biochem. Biophys. Res. Commun.* **199,** 612–618
17. Straub, R. E., Frech, G. C., Joho, R. H., and Gershengorn, M. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9514–9518
18. Ogawa, N., Yamawaki, Y., Kuroda, H., Nukina, I., Ota, Z., Fujino, M., and Yanaihara, N. (1982) *Peptides* **3,** 669–677
19. Sharif, N. A., and Burt, D. R. (1984) *J. Neurochem.* **42,** 209–214
20. Gershengorn, M. C., and Thaw, C. N. (1991) *Endocrinology* **128,** 1204–1206
21. Perlman, J. H., Thaw, C. N., Laakkonen, L., Bowers, C. Y., Osman, R., and Gershengorn, M. C. (1994) *J. Biol. Chem.* **269,** 1610–1613
22. Perlman, J. H., Laakkonen, L., Osman, R., and Gershengorn, M. C. (1995) *Mol. Pharmacol.* **47,** 480–484
23. Ashworth, R., Yu, R., Nelson, E. J., Dermer, S., Gershengorn, M. C., and Hinkle, P. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 512–516
24. Matus-Leibovitch, N., Nussenzveig, D. R., Gershengorn, M. C., and Oron, Y. (1995) *J. Biol. Chem.* **270,** 1041–1047
25. Limbird, L. E. (1986) *Cell Surface Receptors: A Short Course on Theory and Methods*, pp. 1–49, Martinus Nijhoff Publishing, Boston
26. Lee, C., Gardella, T. J., Abou-Samra, A.-B., Nussbaum, S. R., Segre, G. V., Potts, J. T., Jr., Kronenberg, H. M., and Jüppner, H. (1994) *Endocrinology* **135,** 1488–1495
27. Perlman, J. H., Laakkonen, L., Osman, R., and Gershengorn, M. C. (1994) *J. Biol. Chem.* **269,** 23383–23386