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Effect of Solubilization of Porcine Thyrotropin (TSH) Receptor on TSH Binding and on Radio-Receptor Assay for Anti-TSH Receptor Antibodies

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The effect of solubilization of porcine thyrotropin receptor (TSHR) on TSH binding and on the radio-receptor assay for anti-TSHR antibodies was examined. After TSHR solubilization with 1% dodecylpolyethyleneglycoether, TSH binding affinity was increased, from Kd = 1.15nM to 0.45nM, and TSH binding capacity was slightly increased, from 0.15nM to 0.19nM. With a particulate membrane suspension from thyroid cells, blocking of TSH binding to the membrane suspension by anti-thyrotropin receptor antibody was observed only for thyroid stimulation blocking antibody (TSBAb), not for thyroid-stimulating antibody (TSAb). After the solubilization of TSHR, both TSBAb and TSAb blocked TSH-binding to the solubilized TSHR. We speculate that TSAb interacts with the TSHR in the native conformation without interfering with TSH binding, and that after the solubilization, any anti-TSHR antibody interferes with TSH-binding due to the conformational change in TSHR. With these particulate thyroid cell membrane preparations, we can detect only TSBAb by the radio-receptor assay. © 1998 Academic Press

The thyrotropin receptor (TSHR) belongs to a specific subfamily of G protein-coupled receptors with seven transmembrane-spanning regions, and has a large extracellular domain that is the site for high-affinity TSH binding (1, 2). Autoantibodies to TSHR play an essential role in the pathogenesis of Graves' disease and primary atrophic hypothyroidism (3). In Graves' disease, thyroid-stimulating antibody (TSAb), which binds to TSHR and stimulates thyroid hormone production of thyrocytes, causes hyperthyroidism. In primary atrophic hypothyroidism, thyroid stimulation blocking antibody (TSBAb), which binds to TSHR and inhibits the action of thyrotropin (TSH), induces thyroid atrophy and hypothyroidism. Increasing evidence suggests that the binding sites of TSAb to TSHR lie apart from those of TSH or TSBAb (4-6). Therefore, TSAb may not compete with TSH for binding of native TSHR. However, the conventional radio-receptor assay for anti-TSHR antibody (TRAb), which measures inhibition of TSH-binding to a solubilized thyroid cell membrane preparation by anti-TSHR antibodies, referred to as thyrotropin binding inhibitory immunoglobulin (TBII), detects both TSAb and TSBAb together and cannot distinguish between them (7). TSHR might become partially denatured in the solubilized thyroid membrane preparation of the conventional TBII assay, and TSAb can then inhibit the binding of TSH to the denatured TSHR. Various solubilization effects of other receptors on ligand binding have been reported: loss or decrease of binding affinity (8-10), enhancement of agonist binding affinity (11, 12), or change in subtype-specificity of ligand binding (13). The effect of solubilization of TSHR on TSH or TRAb binding is not well understood. In this study we examined TSH-binding to particulate or solubilized porcine TSHR, and compared TBII activity in the radio-receptor assays using these two membrane preparations.

MATERIALS AND METHODS

Serum Samples

Pooled TSAb-positive IgG (TSAb=390%, TSBAb= −1.5%) and pooled TSBAb-positive IgG (TSAb=89%, TSBAb=95.6%) were prepared using a protein A column (Ampure PA: Amersham Japan Co., Japan) from nine TSAb-positive or seven TSBAb-positive autoimmune thyroid disease patients. For the comparison of the new radio-receptor assay with TSAb or TSBAb bioassays, 37 sera with positive TSAb and/or TSBAb from patients with autoimmune thyroid disease were studied. To obtain the immunoglobulin (Ig) fraction, 50 μl of serum was precipitated with 500μl of 0.15 M NaCl solution containing 15% polyethylene glycol 6000 (PEG) at 2,000×g for 20 min-

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utes. The pellets were suspended in 100 μl of assay buffer A containing 10 mM Tris-HCl buffer, pH 7.4, 0.1% bovine serum albumin and 0.05 M NaCl.

Preparation of Porcine Thyroid Particulate Membrane Suspension

Twenty grams of a porcine thyroid gland were cut in to small pieces and washed with physiological saline, and then 70 ml of preparation solution [10 mM Tris-HCl buffer, pH 7.4, 0.93 g/l of iodoacetamide (Wako Pure Chemical Co. Japan), 0.01 g/L leupeptin (Funakoshi Co. Japan), 0.01 g/L soybean trypsin inhibitor (Sigma Chemical Co. USA), 200 kIU/mL aprotinin (Union Chemical Co. Japan) and 0.15 M NaCl] was added. The mixture was homogenized 4 times for 15 sec with a Polytron homogenizer, and then 4 times at 1,500 rpm with a Potter homogenizer. The homogenate was centrifuged at 500×g (4°C; 30 min) and the supernatant was centrifuged at 100,000×g (4°C; 30 min). The pellet was resuspended in 20 ml of preparation solution containing 0.15 M NaCl, homogenized 3 times for 10 sec with a Polytron homogenizer, and centrifuged at 1,500×g (4°C; 30 min). The supernatant was used as porcine thyroid particulate membrane suspension, and stored at −80°C.

Preparation of Solubilized Porcine Thyroid Membrane

Dodecylpolyethyleneglycoether (Thesit: Boehringer Mannheim GmbH. Germany) was added to porcine thyroid membrane suspension (final concentration, 1%). The mixture was gently stirred for 1 hr at 4°C, and centrifuged at 100,000×g for 30 min, and the supernatant was used as solubilized porcine TSHR solution. The solution was stored at −80°C until use for TBII assays.

TSH Binding Analysis

To examine the effect of dodecylpolyethyleneglycoether on TSHR solubilization, thyroid membrane solution was prepared with or without dodecylpolyethyleneglycoether according to the above procedure, and the binding of [^{125}I]-TSH to the thyroid membrane preparations was examined. Labeling of TSH with radioisotope was performed as previously described (7). The dissociation constant between [^{125}I]-TSH and the membrane solution was determined by Scatchard analysis. The dissociation velocity was measured by determining the dissociation of [^{125}I]-TSH from the membrane solution in the presence of 10 μg of unlabeled TSH (5000-fold excess compared to labeled TSH). TSH binding was calculated as percent binding as follows;

$$\left[\frac{\text{(Radioactivity of bound fraction)} - \text{(Radioactivity of nonspecific binding)}}{\text{(Total radioactivity of [}^{125}\text{I]-TSH)}}\right] \times 100\ (\%)$$

Measurement of TBII with Porcine Thyroid Particulate or Solubilized Membrane

One hundred μl of immunoglobulin fraction was mixed with 50 μl of porcine thyroid particulate membrane suspension, or 100 μl of serum diluted 2-fold in assay buffer was mixed with 50 μl of solubilized porcine thyroid membrane solution, and these mixtures were incubated for 15 min at room temperature. Then, 50 μl of [^{125}I]-labeled bovine TSH (10000 cpm) was added. After incubation for 1 hr at 37°C, 550 μl of assay buffer A and 750 μl of 30% PEG in 1M NaCl were added. The mixtures were centrifuged at 2000×g for 20 min and radioactivity in the pellets was measured. TBII activity, expressed as the percentage of inhibition of [^{125}I]-TSH binding, was calculated as follows:

$$\left[1 - \frac{\text{[}^{125}\text{I]-TSH bound in the presence of sample IgG}}{\text{[}^{125}\text{I]-TSH bound in the presence of normal pooled IgG}}\right] \times 100\ (\%)$$

Measurement of TSAb and TSBAb with FRTL-5 Cells

a. Serum immunoglobulin preparation. Two hundred μl of serum was precipitated with 800 μl of 0.15 M NaCl solution containing 18% polyethylene glycol 6000 (PEG) at 2,000×g for 20 min. The pellets were suspended in 200 μl of assay buffer B (pH 7.4) containing 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1% glucose, 1.0% bovine serum albumin, 100 μg/ml streptomycin and 100 U/ml penicillin.

b. Preparation of FRTL-5 cells. Rat thyroid cell line FRTL-5 was maintained in 6H medium [Ham's F12K medium containing 5% fetal bovine serum, TSH (10 mU/ml), transferrin (5 μg/ml), insulin (10 mg/ml), somatostatin (10 mg/ml), cortisone (10 nM), and glycyl-L-histidyl-L-lysine acetate (10 ng/ml)] medium. FRTL-5 cells were harvested and 2×10^4 cells per well were seeded into a 96-well culture plate. After 6 days of incubation in 6H medium, cells were incubated in 5H medium (6H medium without TSH) for 4 days and used for TSAb or TSBAb assays.

c. Measurement of thyroid stimulating antibody (TSAb). After washing the above cells with assay buffer B, 100 μl of sample immunoglobulin fraction was added to these cells and incubated for 3 hr at 37°C in assay buffer B. The cAMP concentration in the supernatant was measured with a commercially available radioimmunoassay kit (Yamasa Shoyu Co. Ltd., Chiba, Japan). TSAb activity was expressed as the percent increase in cAMP level in comparison to normal pooled immunoglobulin (14). The normal cut-off value of TSAb was <140%.

d. Measurement of thyroid stimulation blocking antibody (TSBAb). After washing the cells with assay buffer B, 100μl of sample Ig fraction and bovine TSH were added (the final concentration of TSH was 10 μU/mL). The cells were incubated for 3 hr at 37°C in assay buffer B. The cAMP concentration in the supernatant was measured with a commercially available radioimmunoassay kit (Yamasa Shoyu). TSBAb activity, expressed as the percent inhibition of cAMP release, was calculated as follows (14);

$$\left[1 - \frac{\text{TSH-induced cAMP increase in the presence of sample IgG}}{\text{TSH-induced cAMP increase in the presence of normal pooled IgG}}\right] \times 100\ (\%)$$

The normal cut-off value of TSAb was <40%.

RESULTS

Effect of Solubilization of Thyroid Membrane on TSH Binding

The time course of the binding of [^{125}I]-TSH to thyroid membrane was examined (Fig. 1). The rate of TSH binding to unsolubilized particulate membrane suspension gradually increased. On the other hand, the initial binding rate to TSHR solubilized with 1% dodecylpolyethyleneglycoether, was high, but the binding rate decreased gradually during incubation at 37°C. In Scatchard analysis at 37°C for 1 hr incubation, the concentra-

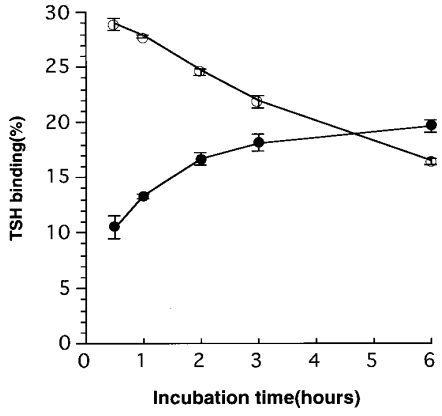

FIG. 1. The effect of dodecylpolyethyleneglycoether on TSH binding to porcine thyroid membrane. [^{125}I]-TSH was incubated with membrane fraction with (○) or without (●) 1% dodecylpolyethyleneglycoether at 37°C for the indicated times. Data are shown as mean±SD.

tion of accessible TSHR in the thyroid particulate membrane suspension was estimated to be 0.15nM, and the dissociation constant, Kd, was 1.15nM (Fig. 2A). When dodecylpolyethyleneglycoether was added to the thyroid membrane suspension, the concentration of accessible TSHR was 0.19nM, and the dissociation constant, Kd, decreased to 0.45nM (Fig. 2B). The rate of [^{125}I]-TSH dissociation in the presence of excess unlabeled TSH was slower for the membrane preparation with dodecylpolyethyleneglycoether solubilization than for that without solubilization (Fig. 3).

Measurement of TBII and Comparison with Bioassays

Anti-TSHR autoantibodies from the patients with autoimmune thyroid disease were analyzed for TBII with particulate or solubilized thyroid membranes. The results were compared with bioassays of TSAb and TSBAb (summarized in Table 1). The samples in group A had TSAb activity and no TSBAbs. In these samples, TBII with the particulate membrane suspension was negative and TBII with the solubilized receptor was positive. The samples in group B had TSBAb activity with or without TSAbs, and the TBII with the particulate membrane suspension was positive, in contrast to the TSAb-positive samples in group A. Thus, TBII with the particulate membrane suspension was positive only in TSBAb-positive samples, whereas TBII with solubilized TSHR solution was positive for both TSAb and TSBAb. The effect of solubilization on the TBII assay was also examined using pooled IgG samples containing only TSAb or TSBAb (Fig. 4). Solubilization of TSHR had an effect only on the TSAb samples (Fig. 4A). The TBII values of TSBAb IgG decreased slightly after solubilization (Fig. 4B).

DISCUSSION

To examine the binding interaction between ligands and their receptors, and to purify the receptors, different receptors have been solubilized by various methods. The receptors solubilized with detergents usually retain their original binding activity, but sometimes change their specificity of ligand binding (13). Interestingly, adrenergic receptors, which are G protein-coupled receptors with seven transmembrane-spanning regions, increase their binding affinity to agonists but not to antagonists after solubilization with digitonin (11, 12). TSHR also belongs to a subfamily of G protein-coupled receptors with seven transmembrane-spanning regions; however, the effect of TSHR-solubilization on ligand binding is poorly understood. In this study, we found that solubilization of TSHR increased the TSH binding affinity and capacity, and changed the characteristics of antibody binding. The decreased rate of dissociation of labeled TSH bound to solubilized

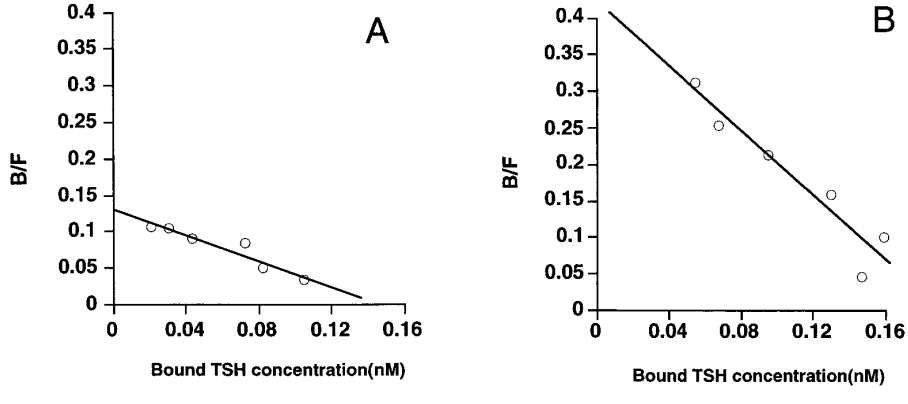

FIG. 2. Scatchard analysis of TSH binding to particulate thyroid membrane suspension with (B) or without (A) dodecylpolyethyleneglycoether.

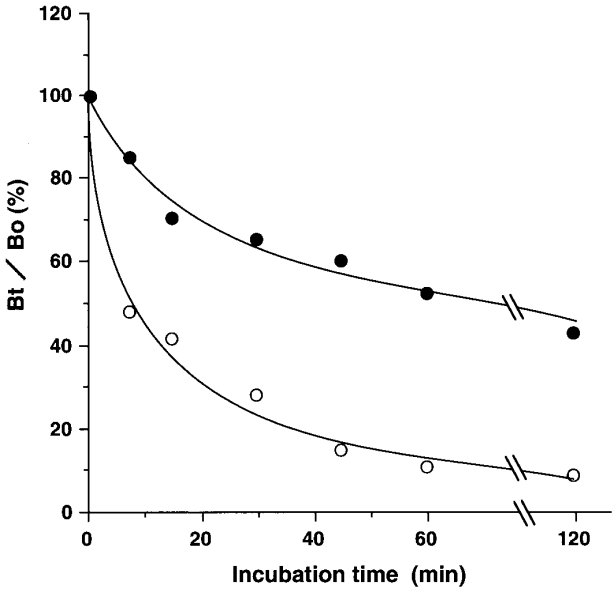

FIG. 3. Dissociation of bound [^{125}I]-TSH from membrane preparation in the presence of excess unlabeled TSH. Each membrane preparation was preincubated with [^{125}I]-TSH at 37°C for 30 min. Then, the membrane preparations were incubated with excess unlabeled TSH at 37°C for 7.5, 15, 30, 45, 60 or 120 min. Residual [^{125}I]-TSH bound to the membrane preparation was expressed as percent in bound [^{125}I]-TSH relative to that immediately after preincubation. The experiments were performed in the absence (open circles) or presence (closed circles) of 1% dodecylpolyethyleneglycoether (DPGE) throughout the procedure.

TSHR is compatible with the previous report by Brennan et al. (15). After solubilization, TSH may become more accessible to TSHR in the membrane solution, wherein TSHR may be somewhat denatured, and the TSH-TSHR complex may be more stable than the complex in the unsolubilized membrane suspension.

The radio-receptor assay for anti-TSHR antibody (TBII), which was developed by Smith et al. (16), made the diagnosis of Graves' disease simple for many cases. Currently solubilized TSHR is widely used due to its increased sensitivity (17), but little attention has been paid to the effect of receptor solubilization on the radio-receptor assay. In this study, only TSBAb was detected with gently prepared particulate thyroid membrane. In the early studies, only a limited number of Graves' sera were examined for TBII using a particulate membrane fraction (18, 19), but positive reaction in the TBII assay could not exclude the possibility of detection of coexisting TSBAb, since TSBAb often coexists with TSAb in Graves' sera (7, 20-22). On the other hand, the unsolubilized membrane fraction of bovine thyroid cells could not detect any TBII in Graves' sera (23) and this result is compatible with our present study. With gently prepared thyroid membrane suspension, wherein TSHR is expected to be in a relatively native conformation, TSAb may bind to a different portion of TSHR than TSH does, and so TSAb may not block TSH binding. On the other hand, TSBAb may bind to TSHR in a way that competes with TSH, and thereby block the effect of TSH.

In patients with both TSAb and TSBAb, it has been difficult to analyze the antibody profile, because TSAb

TABLE 1

Effect of Solubilization of Porcine Thyroid Membrane by Dodecylpolyethylene-Glycoether on Radioreceptor Assay for Anti-TSH Receptor Antibodies

		Porcine thyroid membrane			
		Particulate	Solubilized	FRTL-5 cells	
Group	No.	TBII (%)	TBII (%)	TSAb (%)	TSBAb (%)
A	1	7.6	57.0	167	−20.2
	2	5.1	40.9	208	−14.1
	3	−0.7	46.1	175	−12.1
	4	10.7	31.0	175	−9.7
	5	−2.3	70.0	145	−8.8
	6	1.6	38.1	675	−7.5
	7	6.1	70.2	383	−3.1
	8	−5.0	37.5	140	−2.0
	9	5.1	46.7	192	0.2
	10	0.7	34.6	175	2.3
	11	−7.5	65.3	395	3.1
	12	1.6	33.6	167	4.2
	13	4.7	31.3	171	5.0
	14	7.7	36.1	300	9.9
	15	4.8	52.2	210	11.3
	16	2.1	69.6	346	12.8
	17	6.8	51.2	290	13.1
	18	6.5	75.6	250	13.2
	19	−3.5	27.1	296	14.5
	20	8.9	73.8	171	15.1
	21	1.3	45.0	196	15.5
	22	8.1	50.7	270	18.8
	23	−8.7	47.2	230	22.1
	24	8.1	43.1	500	24.6
	25	11.4	57.7	440	34.7
B	1	25.2	81.8	205	60.0
	2	32.7	87.2	392	61.3
	3	20.5	31.1	235	76.5
	4	78.1	88.3	465	78.1
	5	45.3	82.4	429	80.5
	6	30.4	27.7	95	85.8
	7	88.8	99.1	308	85.9
	8	22.4	65.9	80	86.9
	9	28.2	83.7	160	87.4
	10	56.6	71.7	205	91.6
	11	32.9	34.4	95	92.5
	12	26.1	76.1	385	96.0
C	1	−0.2	−5.7	125	6.1
	2	−9.8	1.7	113	3.8
	3	0.8	−1.3	110	−4.4
	4	9.3	−0.5	90	−3.1
	5	0.0	5.9	105	7.3

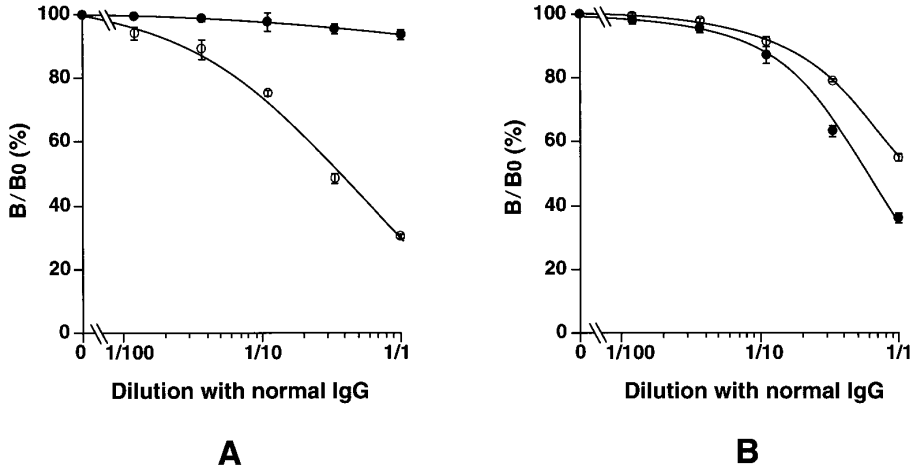

FIG. 4. Effect of solubilization of TSHR on TBII assay. A: Pooled IgG (6mg/ml) from TSAb-positive sera was diluted with normal IgG and incubated with [^{125}I]-TSH and TSHR preparations (●: particulate, ○: solubilized). B: A similar experiment using pooled IgG from TSBAb-positive sera.

and TSBAb present in the same sample cannot be distinguished by the conventional TBII assay. Instead, a complicated bioassay has to be employed to determine the biological activity of the antibodies. The present findings raise the possibility of measuring TSHR antibodies that have TSBAb activity with the simple radioreceptor assay separately, since only TSBAb can interfere with TSH binding and act as TBII with a gently prepared thyroid membrane suspension.

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REFERENCES

1. Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gérard, C., Perret, J., Sande, J., Dumont, J., and Vassart, G. (1989) *Science* **246,** 1620–1622.
2. Nagayama, Y., and Rapoport, B. (1992) *Mol. Endocrinol.* **6,** 145–156.
3. Endo, T., and Onaya, T. (1993) *Endocr. J.* **40,** 625–631.
4. Tahara, K., Ban, T., Minegishi, T., and Kohn, L. D. (1991) *Biochem. Biophys. Res. Commun.* **179,** 70–77.
5. Kosugi, S., Ban, T., Akamizu, T., and Kohn, L. D. (1992) *Mol. Endocrinol.* **6,** 166–180.
6. Dallas, J. S., Desai, R. K., Cunningham, S. J., Morris, J. C., Seetharamaiah, G. S., Wagle, N., Goldblum, R. M., and Prabhakar, B. S. (1994) *Endocrinology* **134,** 1437–45.
7. Watanabe, Y., Tahara, K., Hirai, A., Tada, H., Kohn, L. D., and Amino, N. (1997) *Thyroid* **7,** 13–19.
8. Vasudevan, S., Hulme, E. C., Bach, M., Haase, W., Pavia, J., and Reilander, H. (1995) *Eur. J. Biochem.* **227,** 466–475.
9. Lazard, D., Villageois, P., Briend-Sutren, M. M., Cavaille, F., and Bottari, S. (1994) *Eur. J. Biochem.* **220,** 915–926.
10. Smisterova, J., Ensing, K., de Boer, J., and de Zeeuw, R. A. (1995) *Prep. Biochem.* **25,** 223–251.
11. Pike, L. J., and Lefkowitz, R. J. (1978) *Mol. Pharmacol.* **14,** 370–375.
12. Wikberg, J. E. S., Lefkowitz, R. J., and Caron, M. G. (1983) *Biochem. Pharmacol.* **21,** 3171–3178.
13. Rinken, A. (1995) *J. Pharmacol. Exp. Ther.* **272,** 8–14.
14. Tamaki, H., Amino, N., Kimura, M., Hidaka, Y., Takeoka, K., and Miyai, K. (1990) *J. Clin. Endocrinol. Metab.* **71,** 1382–1386.
15. Brennan, A., Petersen, V. B., Petersen, M. M., Smith, B. R., and Hall, R. (1980) *FEBS Lett.* **111,** 35–38.
16. Smith, B. R., and Hall, R. (1974) *Lancet* **I,** 427–431.
17. Kotulla, P., and Schuleusener, H. (1981) *J. Endocrinol. Invest.* **4,** 155–160.
18. Smith, B. R., and Hall, R. (1974) *FEBS Lett.* **42,** 301–304.
19. Smith, B. R., and Hall, R. (1981) *Methods Enzymol.* **74,** 405–420.
20. Miyauchi, A., Amino, N., Tamaki, H., and Kuma, K. (1988) *Am. J. Med.* **85,** 418–420.
21. Macchia, E., Concetti, R., Carone, G., Borogoni, F., Fenzi, G. F., and Pinchera, A. (1988) *Clin. Endocrinol. (Oxf)* **28,** 147–156.
22. Morgenthaler, N. G., Kim, M. R., Tremble, J., Huang, G. C., Richter, W., Gupta, M., Scherbaum, W. A., McGregor, A. M., and Banga, J. P. (1996) *J. Clin. Endocrinol. Metab.* **81,** 3155–3161.
23. Amir, S. M., Carraway Jr, T. F., and Kohn, L. D. (1973) *J. Biol. Chem.* **248,** 4092–4100.