0026-895X/94/040644-09$03.00/0

MOLECULAR PHARMACOLOGY, 46:644-652, 1994.

Kinetics and Reversibility of Thyrotropin-Releasing Hormone-Stimulated Guanine Nucleotide Exchange in Membranes from GH_4C_1 Cells

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Received February 15, 1994; Accepted July 22, 1994

SUMMARY

To evaluate the role of thyrotropin-releasing hormone (TRH)-stimulated guanine nucleotide exchange in the biphasic cellular responses to TRH, we have examined the kinetics, reversibility, and inhibition by QC120 (an antiserum recognizing the carboxyl terminus of $\alpha_{q/11}$) of TRH-stimulated guanosine-5′-(α-[^{35}S] thio)triphosphate ([^{35}S]GTPαS) binding in membranes from GH_4C_1 cells. Enhanced binding of [^{35}S]GTPαS stimulated by TRH was dose dependent and readily detectable within 8 sec of TRH treatment. Binding measured within the first 20 sec was largely inhibited by QC120, whereas additional binding that accumulated during incubations of 3–6 min was not inhibited by even high concentrations of the antiserum. TRH-stimulated binding was reversible, in that, after membranes were incubated with TRH and [^{35}S]GTPαS, subsequent addition of excess GTP caused exchange of 70–100% of the prebound radioligand. Exchange of TRH-stimulated [^{35}S]GTPαS binding occurred in fast and slow phases, with half-times of <5 sec and 187 sec, respectively. Addition of QC120 before the GTP chase inhibited the fast phase of exchange, whereas reduction of the TRH concentration in the preincubation selectively reduced the magnitude of the slow phase. Neither phase of exchange was affected by prior treatment of cells with pertussis toxin. Our observations indicate that $G_{q/11}$ is rapidly activated by the TRH receptor and that a second, unidentified, G protein is slowly activated by the TRH receptor.

GH_4C_1 cells are a clonal strain of rat pituitary cells that secrete growth hormone and prolactin (1). Secretion and synthesis of prolactin are enhanced by the hypothalamic tripeptide TRH (2), whereas SRIF inhibits prolactin secretion. TRH stimulates rapid biphasic changes in intracellular inositol-1,4,5-trisphosphate levels (3) and $[Ca^{2+}]_i$ (4). The rate of prolactin release closely follows the biphasic changes in $[Ca^{2+}]_i$ (5). In contrast, SRIF decreases $[Ca^{2+}]_i$ in GH_4C_1 cells indirectly by increasing potassium conductance through voltage- and calcium-activated K^+ channels via a mechanism involving an inhibitory G protein (6).

The biochemical and physiological responses to TRH are rapid in onset. Peak increases in $[Ca^{2+}]_i$ are observed within <10 sec with maximum concentrations of TRH, and $[Ca^{2+}]_i$ then falls to a plateau level within ~90 sec (4). The TRH responses are mediated by a 43.5-kDa receptor (7) linked to a pertussis toxin-insensitive G protein that has been shown to be immunologically related to G_q (8). G_q has been shown to have properties that distinguish it from other known G proteins, e.g., exceptionally high affinity for βγ subunits (9), low apparent affinity for GTPγS (10), and insensitivity to activation by GTP in reconstitution assays (10). The GTPase activity of G_q has been shown to be regulated allosterically by its own effector enzyme, phospholipase C-β_1 (11). Thus, knowledge of the details of the coupling between this G protein and a receptor in the membrane environment should promote understanding of the role of this unique G protein in signal transduction.

We have, therefore, developed assays for TRH-stimulated guanine nucleotide exchange that can be used to measure changes occurring in GH_4C_1 membranes on a time scale relevant to the known physiological and biochemical responses to TRH. To demonstrate specificity of these responses, we have also prepared a polyclonal antiserum, QC120, that recognizes the carboxyl terminus of $\alpha_{q/11}$ and prevents its interaction with the TRH receptor. In this report, we utilize these new tools to characterize the rapid TRH-stimulated GTP exchange into

This investigation was supported in part by a research grant from the National Institute of Diabetes and Digestive and Kidney Diseases (Grant DK11011) and by a postdoctoral fellowship (Award PF-3356) from the American Cancer Society to K.D.B.

ABBREVIATIONS: TRH, thyrotropin-releasing hormone; $[Ca^{2+}]_i$, cytosolic free calcium concentration; GTPαS, guanosine-5′-(α-thio)triphosphate; GTPγS, guanosine-5′-(γ-thio)triphosphate; SRIF, somatostatin; VIP, vasoactive intestinal peptide; B_{fast}, fast binding (sites); B_{slow}, slow binding (sites); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HBSS, Hanks' balanced salt solution.

$G_{q/11}$ and to demonstrate the presence and properties of a second TRH-stimulated GTP-binding activity, which is not inhibitable by QC120.

Experimental Procedures

Materials

Tissue culture plasticware was from Falcon Labware (Division of Becton Dickinson, Lincoln Park, NJ). Culture medium and sera were from GIBCO Laboratories (Grand Island, NY). TRH, N^{τ}-methyl-TRH, and SRIF were purchased from Peninsula Laboratories (Belmont, CA). [^{35}S]GTPαS (1000–1300 Ci/mmol) and Aquasol were obtained from NEN Research Products (Boston, MA). The Tris salt of GTP, soybean trypsin inhibitor, leupeptin, pepstatin, β,γ-imidoadenosine 5′-triphosphate, phenylmethylsulfonyl fluoride, iodoacetamide, disodium ATP, (−)-N^6-(*R*-2-phenylisopropyl)adenosine, VIP, and buffer salts were purchased from Sigma Chemical Co. (St. Louis, MO). Glass fiber filters (type G6) were purchased from Fisher Scientific (Pittsburgh, PA). GTPαS was purchased from Amersham (Arlington Heights, IL). Pertussis toxin was from Calbiochem (San Diego, CA). pGlu-His-Pro-NH-$(CH_2)_6$-NH_2 was prepared as described previously (12).

Methods

Cell culture methods. Culture of GH_4C_1 cells has been described in detail (1). Briefly, cells were grown in 100-mm plastic dishes and fed every 3–4 days with Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. Cultures were maintained at 37° in a humidified atmosphere of 5% CO_2/95% air. The cells used were routinely tested to ensure that cultures were free of *Mycoplasma* contamination.

Protein determination. Cell protein concentrations were determined by the Pierce bicinchoninic acid assay (13), using bovine albumin (Sigma product A7906, lot 128F-0785) as the standard.

Antibody preparation. The dodecapeptide CLQLNLKEYNLV was conjugated to bovine albumin using glutaraldehyde and was used to raise antisera in four rabbits (14). Antisera QC119, QC120, QC121, and QC122 were tested for specificity and titer using enzyme-linked immunosorbent assay, immunoprecipitation, and immunoblotting methods. QC120 was selected for this study based on its high titer and low nonspecific background binding. QC120 was partially purified by fractionation with 50% $(NH_4)_2SO_4$, followed by dialysis against 20 mM potassium phosphate, pH 7.2. The protein concentration was adjusted to 20 mg/ml, and the antiserum was stored at −80° until use. Normal rabbit serum was purchased from GIBCO and partially purified by identical methods.

Membrane preparation. Medium was removed from culture dishes by aspiration, and cells were covered with warm (37°) HBSS/EDTA (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM HEPES, 600 μM EDTA, pH 7.4) for 1 min at 25°. The HBSS/EDTA was removed by aspiration and the dishes were incubated at 37° for 4–5 min. Detached cells were suspended in HBSS/Ca^{2+} (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM HEPES, 0.4 mM $CaCl_2$, pH 7.4) and pelleted by centrifugation. All additional steps were performed in cold (4°) buffers. Pooled cells from 36 plates were rinsed in HBSS/Ca^{2+} and pelleted by centrifugation. Cells were then suspended in hypotonic buffer (20 mM MOPS, pH 7.4, 2 mM $MgCl_2$, 1 mM disodium EDTA, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 30 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml iodoacetamide) for 10 min, followed by homogenization with 25 strokes of a 25-ml glass/Teflon Dounce homogenizer. The crude homogenate was centrifuged for 6 min at 750 × *g* to remove nuclei and unbroken cells. To the crude membranes, 4.5 M KCl and 20% digitonin were added to final concentrations of 200 mM and 0.1%, respectively, and the membranes were further dispersed using 15 strokes of the homogenizer. The membranes were then pelleted by centrifugation at 12,000 × *g* for 10 min and were resuspended in buffer A (1.7 M sucrose, 50 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 1 mM potassium EGTA, 5 μg/ml pepstatin, 5 μg/ml leupeptin; density, 1.22 g/ml). Portions of this suspension were transferred to ultracentrifuge tubes and layered with equal volumes of buffer B (same as buffer A but with 1.38 M sucrose; density, 1.18 g/ml) and buffer C (same as buffer A but containing 1.30 M sucrose; density, 1.16 g/ml). The membranes were centrifuged in a swinging bucket rotor (Beckman SW41) for 30 min at 70,000 × *g*. The pellicle at the buffer B/C interface was collected, diluted 10-fold into buffer (20 mM MOPS, pH 7.4, 2 mM $MgCl_2$, 1 mM EGTA), and centrifuged at 12,000 × *g* for 15 min. The pelleted membranes were suspended in assay buffer (50 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 1 mM potassium EGTA, 1 mM dithiothreitol, 5 μg/ml pepstatin, 5 μg/ml leupeptin) to approximately 0.5 mg of protein/ml, divided into aliquots, and frozen at −70° until ready for use. Pertussis toxin-treated membranes were prepared by incubation of cells with 100 ng/ml pertussis toxin for 16 hr before harvesting and membrane preparation as described above. Efficacy of toxin treatment was ascertained by complete inhibition of SRIF-stimulated GTPαS binding.

Assay of stimulated [^{35}S]GTPαS binding. Thawed membranes were diluted into assay buffer at 4°. Aliquots (100 μl, about 20–30 μg of protein) were transferred to vials and warmed to ambient temperature for 8 min. Binding of [^{35}S]GTPαS was initiated by addition of 300 μl of binding cocktail containing 1.33 mM sodium ATP and [^{35}S]GTPαS, with or without TRH or SRIF to attain the final concentrations indicated for each experiment. After the times indicated for each experiment, 360 μl of the reaction mixture were applied to a glass fiber filter (1.5-μm pore size), and the filter was rinsed with 15 ml of ice-cold rinse buffer (5 mM MOPS, pH 7.2, 0.5 mM EDTA). Filters were immersed in scintillation fluid, and retained radioactivity was determined by scintillation counting. Stimulated [^{35}S]GTPαS binding is presented as total binding in the presence of agonist minus basal binding. Standard deviations of this difference were calculated by error propagation methods. To assay the effects of immune sera on stimulated [^{35}S]GTPαS binding, cold membranes were combined with immune serum as described for individual experiments and were preincubated at room temperature for 10 min before initiation of the binding reaction. Longer preincubation times gave no increase in the apparent potency of the immune serum QC120.

To measure the competitive K_d of GTP or GTPαS for basal and TRH-stimulated binding sites, assays were prepared as described, except that GTP or GTPαS was added to final concentrations of up to 100 μM, and the reactions were incubated for 3 or 10 min. K_d values were obtained from plots of basal or TRH-stimulated binding versus competing nucleotide concentration.

[^{35}S]GTPαS exchange assay. Membranes (approximately 100 μg of protein in 0.6 ml) were suspended in cold (4°) assay buffer (50 mM MOPS, pH 7.4, 5 mM $MgCl_2$, 1 mM potassium EGTA, 1 mM dithiothreitol, 5 μg/ml pepstatin, 5 μg/ml leupeptin) and warmed to ambient temperature for 8 min. Binding cocktail ([^{35}S]GTPαS and ATP, with or without TRH, in 1.9 ml of assay buffer) was added to achieve final concentrations of 1.3 nM [^{35}S]GTPαS and 1 mM ATP, without or with 3.5 μM TRH. After incubation for the time interval indicated for each experiment, 0.4 ml of the reaction was removed, and a small volume (5–10 μl) of concentrated GTP solution (sufficient to achieve final concentrations as indicated for each experiment) was applied to the lip of the reaction tube. To initiate the exchange reaction, the first aliquot was applied to a glass fiber filter and, simultaneously, the remaining reaction was agitated to introduce and mix in the GTP. Additional aliquots (0.4 ml) were filtered at intervals thereafter. All filters were rinsed once with 15 ml of ice-cold rinse buffer (5 mM MOPS, pH 7.2, 0.5 mM EDTA). Rinsing was complete within 1 sec of application of the sample to the filter. To observe the effects of immune sera on the exchange of TRH-stimulated [^{35}S]GTPαS binding, antiserum (1/600) was added 40 sec after initiation of the binding reactions, and exchange was initiated after 140 sec of additional incubation in the presence of the serum.

Statistical procedures. Stimulated binding was calculated as the difference between total binding in the presence of agonist and basal

binding in the absence of agonist. The variance of the stimulated binding was calculated as the sum of the experimental variances of total and basal binding. Nonlinear regression analysis of exchange curves was performed using the Levenberg-Marquardt algorithm as provided in SigmaPlot (Jandel Scientific, Corte Madera, CA). Regression results were not found to be sensitive to the choice of initial parameters within a reasonable range, indicating that modeling of the data was not adversely affected by local regression minima.

Results and Discussion

Dose-response curves for TRH-stimulated GTPαS binding. TRH stimulated, in a concentration-dependent manner, a rapid increase in GTPαS binding above the basal level (Fig. 1, *top*). Measured 20 sec after addition of TRH, GTPαS binding was half-maximally stimulated by 50 nM TRH. After a 3-min incubation, the observed EC_{50} for TRH decreased to 11 nM (Fig. 1, *bottom*). The magnitude of the increase in specific binding stimulated by TRH was typically 30–50% above basal binding, with higher values (up to 80% above basal binding) being observed at short incubation times. The dose-response curves were shifted rightward by inclusion in the incubations of chlordiazepoxide (data not shown), a known TRH receptor antagonist that is competitive with TRH (15), confirming that the effect was mediated by the TRH receptor. GH_4C_1 cells also possess SRIF receptors, which are coupled to a pertussis toxin-sensitive G protein (16). After a 3-min incubation, SRIF stimulated GTPαS binding to a similar maximum extent as did TRH, with an EC_{50} of 92 nM (Fig. 1, *bottom*). The dose-response curve for TRH was unchanged when evaluated in the presence of a maximal dose (10 μM) of SRIF (data not shown), demonstrating that these two hormones stimulate binding to unique G proteins. VIP, which induces prolactin secretion via activation of G_s (17), gave no response in these assays. We conclude that the action of TRH on guanine nucleotide exchange in these membrane preparations is rapid, consistent with the known physiological actions of TRH. Except where noted, subsequent experiments utilized maximal (3.5 μM) TRH concentrations.

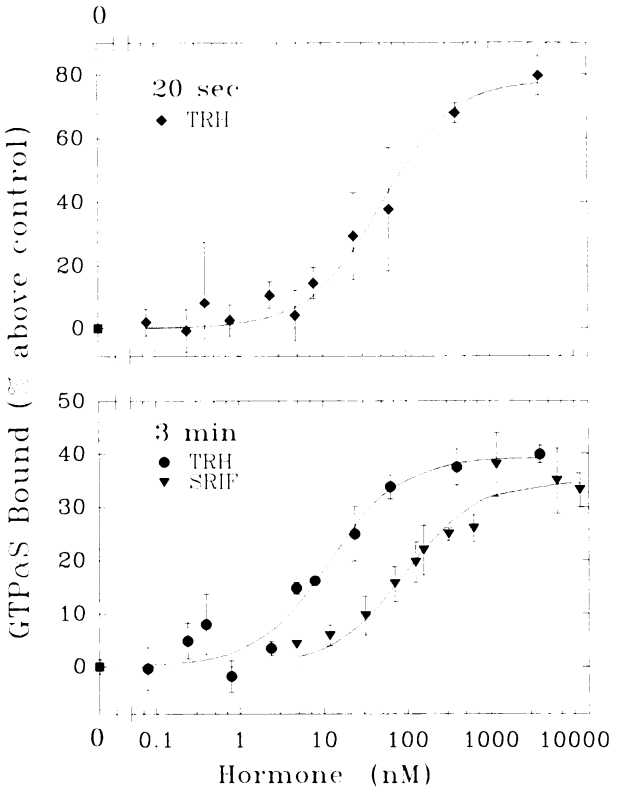

Fig. 1. Dose-response curves for TRH-stimulated and SRIF-stimulated GTPαS binding. GH_4C_1 cell membranes were incubated with 1 nM [^{35}S]GTPαS and increasing concentrations of TRH or SRIF, as described in Experimental Procedures. After 20 sec (*top*) or 3 min (*bottom*), reaction mixtures were applied to filters and membrane-bound [^{35}S]GTPαS was evaluated by scintillation counting. Each *point* gives the mean value; *vertical bars*, standard deviation of triplicate measurements. *Smooth curves*, best fits by nonlinear regression to the expression $B = B_{max}[\text{agonist}]/([\text{agonist}] + EC_{50})$. EC_{50} values were 11.5 nM (TRH, 3 min), 50 nM (TRH, 20 sec), and 92 nM (SRIF, 3 min).

Characterization of QC120, a polyclonal antiserum reactive against $\alpha_{q/11}$. Rabbit antiserum against the carboxyl-terminal dodecapeptide of $\alpha_{q/11}$ (QC120) was prepared as described in Experimental Procedures. Fig. 2 demonstrates the use of QC120 in immunoprecipation and immunoblotting reactions. QC120 immunoprecipitated a 42-kDa band that was not precipitated by normal rabbit serum or when the $\alpha_{q/11}$ carboxyl-terminal peptide was included in the immunoprecipitation reaction (Fig. 2, *top*). QC120 was thus able to recognize the native $\alpha_{q/11}$ proteins. Although QC120 is expected to react with both the α_q and α_{11} proteins (with molecular masses of 42 and 43 kDa, respectively), these two bands would not be resolved using the electrophoresis conditions employed in Fig. 2, *top*. Among other members of the G_q family, $G\alpha_{14}$ has a carboxyl-terminal sequence that is homologous to that of $G\alpha_{q/11}$, whereas the carboxyl termini of $G\alpha_{15/16}$ are distinct (18–20). Both $G\alpha_q$ and $G\alpha_{11}$ are expressed in GH_3 cells, a close relative of GH_4C_1 cells, but none of the other members of the $G\alpha_{q/11}$ family were detected in GH_3 cells using subtype-specific antisera (21).

The specificity of QC120 for $G_{q/11}$ was demonstrated by Western blotting (Fig. 2, *bottom*). QC120 recognized a 42-kDa band from GH_4C_1 cell membranes that comigrated with the band recognized by WO82, an antibody raised against an internal region of the $G\alpha_q$ protein (22). Under optimal conditions, the 42-kDa band recognized by QC120 was resolved into a doublet of 42 and 43 kDa (data not shown). The band recognized by QC120 was clearly separated from $G\alpha_s$, $G\alpha_{i1,2}$, and $G\alpha_{i3,o}$, which were identified using antibodies RM/1 (23), AS/7 (24), and EC/2 (23), respectively. QC120 cross-reacted weakly with several other proteins. Recognition of a 45-kDa doublet and a 38-kDa singlet was specifically blocked by the free carboxyl-terminal dodecapeptide. QC120 showed no reactivity with purified recombinant $G\alpha_s$ under conditions in which RM/1 reacted strongly (data not shown). We conclude that QC120 selectively recognizes $G\alpha_{q/11}$ and that there are at least two other proteins in GH_4C_1 membranes that are immunologically related to $G\alpha_{q/11}$.

QC120 inhibition of rapid TRH-stimulated [^{35}S]GTPαS binding. Fig. 3 illustrates the effects of increasing concentrations of QC120 on TRH-stimulated [^{35}S]GTPαS binding measured after 15 sec (Fig. 3, *top*), 3 min (Fig. 3, *bottom*), or 6 min (Fig. 3, *middle*). At a dilution of 1/60, QC120 inhibited 85% of the rapid response to TRH (Fig. 3, *top*). In incubations of 3- or 6-min duration, a large fraction of the total TRH-stimulated binding was not inhibited by QC120, even at a high (1/12) antibody concentration (Fig. 3, *middle* and *bot-*

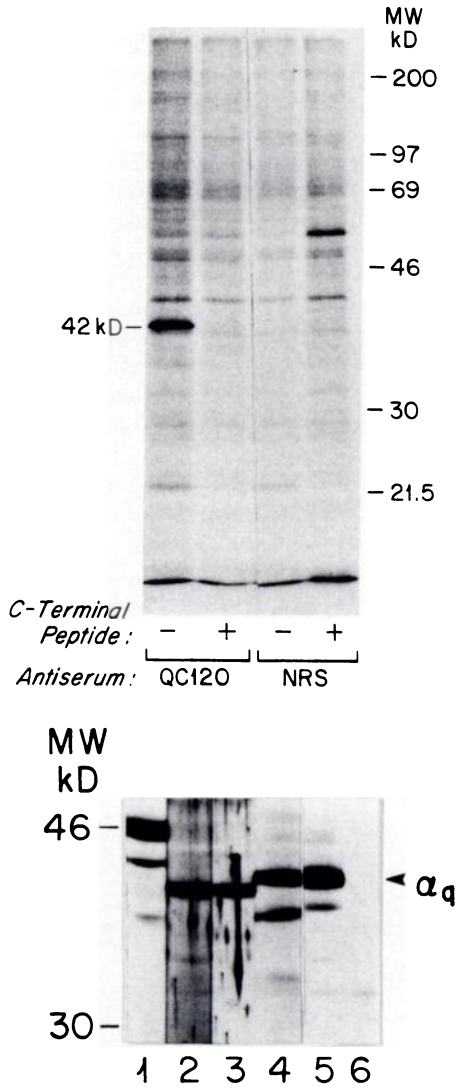

Fig. 2. Specific recognition of a 42-kDa protein by QC120, an anti-$G_{\alpha q/11}$ antibody. *Top*, [^{35}S]methionine-labeled GH_4C_1 cell membranes were solubilized with 1% Nonidet P-40. QC120 was added at 1/1000 dilution and carboxyl-terminal $\alpha_{q/11}$ peptide, when present, was added at 1 μg/ml. Antibody-antigen complexes were recovered by goat anti-rabbit antibody on polyacrylamide beads. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by fluorography. *NRS*, normal rabbit serum. *Bottom*, GH_4C_1 cell membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was performed using the enhanced chemiluminescence system (Amersham). Identical lanes were blotted with various G protein antibodies. *Lane 1*, 1/5000 RM/1 (anti-$G_{\alpha s}$); *lane 2*, 1/1000 AS/7 (anti-$G_{\alpha i1,2}$); *lane 3*, 1/1000 EC/2 (anti-$G_{\alpha i3}$); *lane 4*, 1/400 WO82 (anti-$G_{\alpha q}$); *lane 5*, 1/10,000 QC120; *lane 6*, 1/10,000 QC120 plus $\alpha_{q/11}$ carboxyl-terminal peptide at 1 μg/ml.

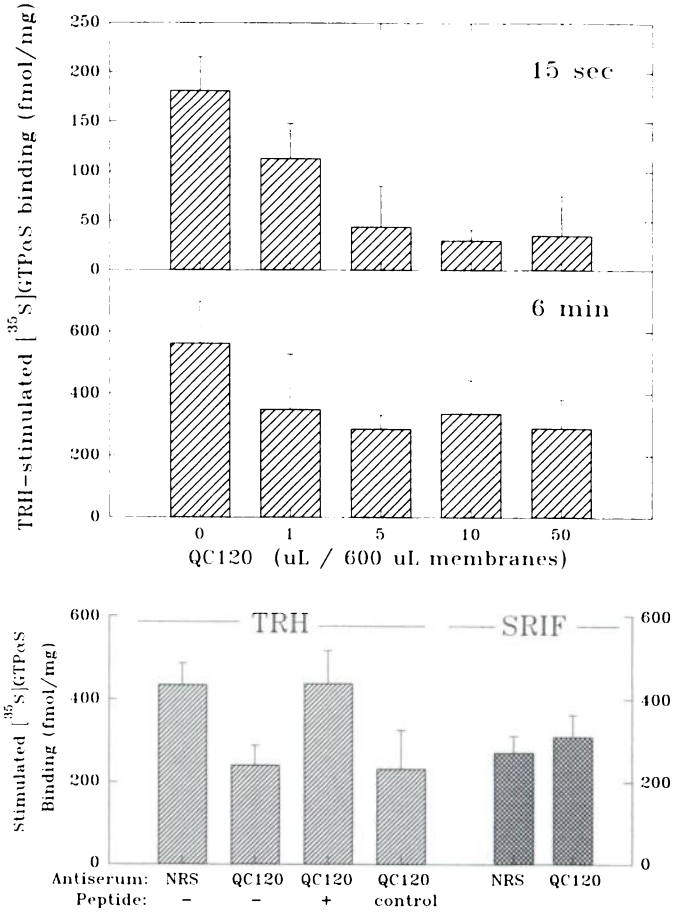

Fig. 3. Specific inhibition by QC120 of rapid TRH-stimulated [^{35}S]GTPαS binding. *Top* and *middle*, QC120 was preincubated with GH_4C_1 membranes at the indicated concentrations, in a total volume of 600 μl. Sufficient normal rabbit serum was added to the reaction vials to achieve equal serum concentrations in all samples. After 10 min, binding cocktail with or without TRH was added to final concentrations of 1 nM [^{35}S]GTPαS, 1 mM ATP, and 3.5 μM TRH, in a total volume of 800 μl. After 15 sec (*top*) or 6 min (*middle*), aliquots were applied to glass filters and bound [^{35}S]GTPαS was evaluated by scintillation counting. *Bottom*, membranes were incubated for 10 min with normal rabbit serum (*NRS*), QC120, or QC120 preincubated with its epitope peptide or with a control peptide, as indicated. The final dilution of all sera was 1/60. TRH- or SRIF-stimulated binding were evaluated using assays of 3-min duration. *Error bars*, standard deviation observed between triplicate samples.

tom). The effects of QC120 were blocked by preincubation of the antibody for 16 hr with its epitope dodecapeptide (Fig. 3, *bottom*), whereas preincubation with a control peptide of irrelevant sequence was without effect. QC120 had no effect on SRIF-stimulated [^{35}S]GTPαS binding (Fig. 3, *bottom right*). If the incomplete inhibition of TRH-stimulated binding by high concentrations of QC120 were due to leakiness of its action, then we would predict that the QC120-inhibitable binding would decrease with time. In contrast, Fig. 3 demonstrates that QC120 inhibited 150–200 fmol/mg of binding whether measured at 15 sec, 3 min, or 6 min. We conclude that the QC120-resistant binding represents binding to a G protein immunologically distinct from $G_{q/11}$.

Exchange of basal and TRH-stimulated GTPαS binding. To determine whether the GTPαS binding stimulated by TRH was reversible, we preincubated membranes with 1.3 nM [^{35}S]GTPαS for 3 min in the absence or presence of 3.5 μM TRH, added a concentrated aliquot of GTP, and observed the exchange of total prebound radioligand (Fig. 4, *top*). Of the portion of binding specifically stimulated by TRH (Fig. 4, *bottom*), exchange sometimes proceeded to completion (as shown), although occasionally up to 25% of the TRH-stimulated counts were refractory to exchange (data not shown). This variable extent of reversibility may have involved proteolysis, because the degree of irreversibility varied between membrane preparations and could be partially ameliorated by

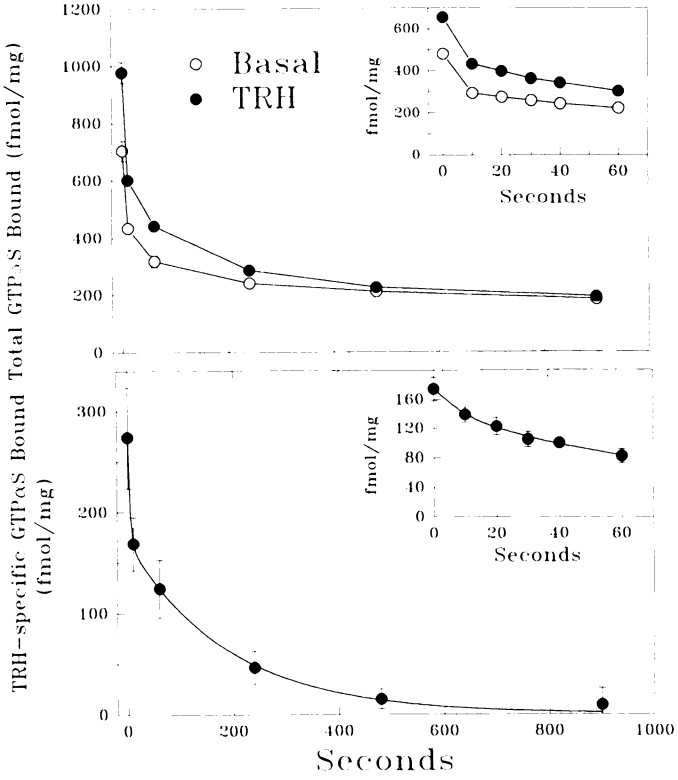

Fig. 4. Exchange of prebound [^{35}S]GTPαS from GH_4C_1 membranes by added GTP. Membranes from GH_4C_1 cells were incubated with 1.3 nM [^{35}S]GTPαS for 3 min in the absence or presence of 3.5 μM TRH. GTP was then added to a final concentration of 1 mM, and remaining bound radioligand was measured at time intervals thereafter. *Top*, exchange of total GTPαS bound in the absence of TRH (○) or in the presence of TRH (●). *Top*, *inset*, results from a separate experiment, with measurements made during the first 1 min after addition of excess GTP. *Bottom*, exchange of specific TRH-stimulated binding (i.e., total binding minus basal binding) from GH_4C_1 membranes. *Bottom*, *inset*, rapid exchange of specific TRH-stimulated binding measured in an independent experiment (see *top*, *inset*). *Error bars*, standard deviation observed between triplicate samples; when not shown, they lie within the symbols.

increased concentrations of protease inhibitors in the assays (data not shown).

Fig. 4, *insets*, illustrates the exchange process observed on a faster time scale. The exchange of the TRH-stimulated prebound radioligand was modeled as a biphasic exponential decay by using eq. 1.

$$B = B_{fast}e^{-k_{fast}t} + B_{slow}e^{-k_{slow}t} \quad (1)$$

Applying this model function to 12 independent measurements of the exchange of TRH-stimulated [^{35}S]GTPαS binding, we determined means ± standard deviations of rate constants as follows: fast exchange process, $k_{fast} \geq 0.13 \pm 0.05$ sec^{-1} ($t_{½} < 5$ sec); slow exchange process, $k_{slow} = 3.7 \pm 2.5 \times 10^{-3}$ sec^{-1} ($t_{½} =$ 187 sec). In other experiments, we have observed that the concentrations of GTP required to exchange B_{fast} and B_{slow} half-maximally were 600 nM and 40 nM, respectively, and that the concentration of GTP used in subsequent experiments (14 μM) was sufficient to achieve maximal exchange of both activities (data not shown).

QC120 inhibition of exchange of B_{fast}. Because the fast exchange process was often nearly complete before our first measurement, it was essential to address whether it might arise as an artifact of the assay. Expecting that rapid guanine nucleotide exchange might correlate with $G_{q/11}$, we incubated GH_4C_1 membranes with 1 nM [^{35}S]GTPαS for 40 sec, added QC120 (1/60), continued the incubation for 140 sec, then added excess GTP, and measured the exchange of prebound [^{35}S]GTPαS during the next 60 sec. Fig. 5, *top*, shows that the rapid phase of exchange was blocked by QC120, and this phase was not affected either by normal rabbit serum or by QC120 that had been preincubated with its epitope peptide, indicating that the rapid phase of exchange was a process specifically involving $G_{q/11}$.

Selective reduction of B_{slow} by reduced agonist concentrations. Searching for additional distinctions between the rapid and slow phases of TRH-stimulated binding, we modified the exchange assay such that membranes were preincubated for 90 or 180 sec with 1.3 nM [^{35}S]GTPαS and a submaximal

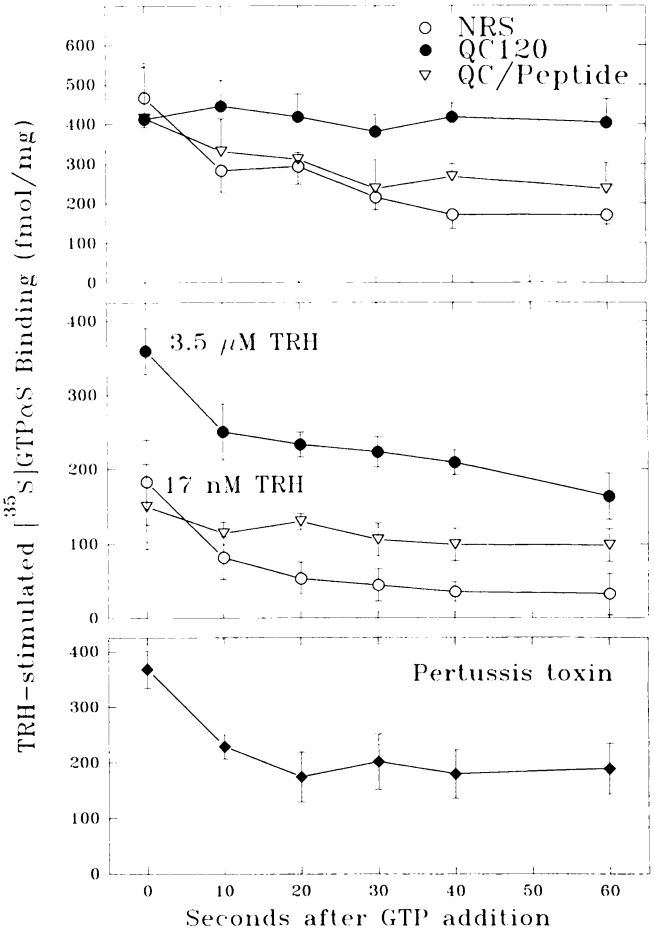

Fig. 5. Effects of QC120, low agonist concentration, and pertussis toxin on exchangeable TRH-stimulated [^{35}S]GTPαS binding. *Top*, membranes were preincubated with 1.3 nM [^{35}S]GTPαS for 40 sec, and normal rabbit serum (*NRS*), QC120, or neutralized QC120 was added to a final 1/60 dilution. After 140 sec of further incubation, GTP was added to a final concentration of 1 mM, and exchange of TRH-stimulated [^{35}S]GTPαS was observed during the next 60 sec. *Middle*, membranes were incubated with 1.3 nM [^{35}S]GTPαS for 3 min in the presence of 3.5 μM TRH (●) or 17 nM TRH (○), and exchange of specifically bound nucleotide was observed after addition of GTP plus TRH to final concentrations of 14 and 3.5 μM, respectively. In one experiment (▽), binding was stimulated by 17 nM TRH but no additional TRH was added during the exchange reaction. *Bottom*, cells were treated with 100 ng/ml pertussis toxin for 16 hr before harvesting and preparation of membranes and measurement of exchangeable TRH-stimulated binding. *Error bars*, standard deviation observed between triplicate samples.

concentration of TRH (17 nM) (see Fig. 1). Additional TRH was then added with the GTP chase to achieve a final concentration of 3.5 μM. The results obtained using this modified assay are summarized in Fig. 5, *middle*, and Table 1. When membranes were loaded with [^{35}S]GTPαS in the presence of 3.5 μM TRH, both B_{fast} and B_{slow} activities were observed in quantities consistent with previous experiments. When membranes were loaded in the presence of only 17 nM TRH and exchange was measured in the presence of 3.5 μM TRH, the magnitude of B_{fast} was unchanged but the magnitude of B_{slow} was reduced by 4–5-fold. We repeated these assays using the TRH analog pGlu-His-Pro-NH-$(CH_2)_6$-NH_2, because this analog differs markedly from TRH in its relative potencies for stimulation of prolactin synthesis versus prolactin secretion (25). Like TRH, this analog also stimulated preferential accumulation of B_{fast} when used at lower concentrations (Table 1). We conclude that there is no simple correlation between activation of B_{fast} versus B_{slow} and stimulation of rapid secretion versus synthesis of prolactin.

If the additional TRH was omitted from the GTP chase, the rate of exchange of B_{fast} was reduced by 9-fold (0.012 sec^{-1}, compared with 0.11 sec^{-1} at 3.5 μM TRH) (Fig. 5, *middle*), demonstrating that the rapid exchange of B_{fast} required the presence of a higher concentration of TRH. If we assume that at 17 nM TRH the rate of accumulation of B_{slow} is also one ninth of its maximal value, then we would predict from the rate of accumulation of B_{slow} (see Fig. 6 and eq. 2) that, in preincubations of 90–180 sec, B_{fast} would accumulate to maximal levels, whereas B_{slow} would accumulate to only 15–19% of the amount observed after maximal TRH stimulation. This prediction correlates closely with the level of activation of B_{slow} by TRH observed in Fig. 5 and Table 1. We propose that, at low agonist concentrations, binding to both types of sites is activated, although at a reduced rate. However, because k_{fast} is much greater than k_{slow}, in assays of 1–3-min duration we observe maximal accumulation of radioligand in B_{fast}, with reduced accumulation in B_{slow}.

Knowing that the rate of GTPαS binding stimulated by 17 nM TRH is 9-fold lower than the maximal rate, we may calculate the functional K_d of TRH for its receptor to be 136 nM (i.e., the K_d such that at 17 nM TRH the receptors are one ninth occupied). Notably, this value is greater than both the known K_d of TRH for its receptor in GH_4C_1 membrane preparations (18 nM) (25) and the EC_{50} for stimulation of GTPαS binding measured at either 20 sec or 3 min (Fig. 1). The former discrepancy most likely results from different protocols for equilibrium binding studies (which generally utilize a 1-hr preincubation) and our measurements, which utilize very short incubations. The difference between the functional K_d and the EC_{50} values for accumulation of bound guanine nucleotide illustrates the complexity of EC_{50}. Using the functional K_d of 136 nM and the rate constants for binding of B_{fast} and B_{slow} measured in this study (Fig. 6) and assuming that, under conditions where G protein is present in excess over the amount of receptor, the rate of guanine nucleotide exchange is proportional to the receptor occupancy, we have simulated theoretical binding data that closely resemble the data of Fig. 1 (data not shown). We conclude that data collected after short incubations are essentially of a kinetic nature and that the resemblance of the EC_{50} values to K_d values measured under equilibrium conditions is coincidental.

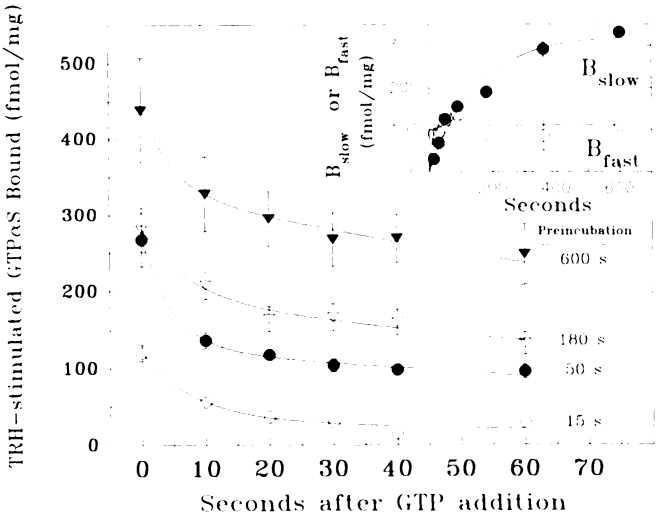

Fig. 6. Rates of accumulation of B_{fast} and B_{slow}. Membranes and 1.3 nM [^{35}S]GTPαS were incubated in assay buffer, in the absence or presence of TRH, for intervals between 15 and 600 sec, GTP was added to a final concentration of 14 μM, and TRH-stimulated [^{35}S]GTPαS binding was measured during the next 60 sec. Data from 30-, 90-, and 360-sec preincubations have been omitted for clarity. *Error bars*, standard deviation among triplicate reactions; when not shown, they lie within the symbols. *Smooth curves*, best fit to eq. 1 by nonlinear regression. *Inset*, B_{fast} and B_{slow} were evaluated by nonlinear regression analysis and are plotted as a function of the preincubation interval. *Error bars*, standard deviation of the regression; when not shown, they lie within the symbols. *Smooth curves*, best fits of B_{fast} or B_{slow} to the expression $B = B_{max}(1 - e^{-kt})$ (eq. 2).

Persistence of biphasic exchange in pertussis toxin-pretreated membranes. Calcium mobilization and prolactin secretion stimulated by TRH persist after pertussis toxin treatment of GH_4C_1 cells (26), whereas responses to SRIF are inhibited by pertussis toxin (27). However, partial inhibition of TRH-stimulated GTPγS binding and a pertussis toxin-sensitive TRH-stimulatable calcium current have been reported (28). To determine whether the B_{slow} was inhibited by pertussis toxin, we prepared membranes from cells that had been treated with the toxin (100 ng/ml) for 16 hr before harvesting. Exchange assays performed using these membranes (Fig. 5, *bottom*) were identical, in both magnitude and time

TABLE 1

Magnitudes of B_{fast} and B_{slow} stimulated by low and high concentrations of TRH or pGlu-His-Pro-NH-$(CH_2)_6$-NH_2 (TRH-NH_2)

Fitting to eq. 1 was used to estimate values for B_{fast} and B_{slow} from the data in Fig. 5 (180-sec incubations) and similar experiments (data not shown) using a shorter (90-sec) incubation interval.

Agonist	Incubation	B_{fast}	B_{slow}
	sec	*fmol/mg*	*fmol/mg*
TRH, 17 nM	90	137	28
TRH, 3.5 μM	90	135	158
TRH, 17 nM	180	139	41
TRH, 3.5 μM	180	109	244
TRH, 17 nM; exchange at 17 nM[a]	180	122 (k_{fast} ~ 0.012 sec^{-1})	28
TRH-NH_2, 70 nM	90	133	14
TRH-NH_2, 50 nM	180	156	59
TRH-NH_2, 5.5 μM	180	205	193

[a] Additional TRH was omitted from the exchange reaction, so that the TRH concentration during exchange remained at 17 nM. B_{fast} and B_{slow} were estimated as 83% (122 fmol/mg) and 17% (28 fmol/mg) of the total binding, respectively, as indicated. Fitting to eq. 1 with these estimates introduced as constants and k_{slow} = 0 yielded k_{fast} = 0.012 sec^{-1} under these conditions.

course, to profiles from control membranes. In contrast, the SRIF-induced binding of [^{35}S]GTPαS was completely abolished in these membranes (data not shown), confirming the effectiveness of toxin treatment. We have also observed that the [^{35}S]GTPαS binding responses to TRH and SRIF are completely additive, i.e., each hormone stimulated an amount of GTPαS binding independently of the presence of the other hormone. This result demonstrates that they each activate binding to a unique set of G proteins and that the QC120-resistant binding stimulated by TRH (B_{slow}) is not related to the pertussis toxin-sensitive G proteins G_i or G_o.

TRH has been reported to weakly stimulate adenylate cyclase in GH_4C_1 cells via activation of G_s (29), but efforts to evaluate the possible involvement of G_s in the slow TRH-stimulated [^{35}S]GTPαS binding response have been hampered by the lack of a suitable positive control response in GH_4C_1 cell membranes. Although it is difficult to rationalize why TRH might stimulate G_s whereas VIP (a prolactin secretagogue that acts via G_s) (17) gave no response, involvement of G_s in the B_{slow} response cannot be ruled out at this time.

Time-dependent accumulation of B_{fast} and B_{slow}. To observe how the magnitudes of B_{fast} and B_{slow} depended on the duration of preincubation of membranes with [^{35}S]GTPαS, we performed a series of exchange reactions in which the preincubation interval was varied from 15 to 600 sec. The exchange profiles of TRH-stimulated GTPαS binding (Fig. 6) were biphasic for all preincubation times, and B_{fast} and B_{slow} levels were estimated by fitting to eq. 1. B_{fast} and B_{slow} levels are plotted as a function of the preincubation interval in Fig. 6, *inset*. Accumulation of each of these activities was modeled using eq. 2.

$$B = B_{max}(1 - e^{-kt}) \quad (2)$$

The B_{fast} level had attained its maximal value of 110 fmol/mg at the time of the first measurement (15-sec preincubation), and we estimate the $t_{1/2}$ to be $\leq$5 sec ($k \geq 0.14$ sec^{-1}), although previous studies suggested that the rate of this process may actually be faster (30). The TRH-stimulated B_{slow} accumulated, with a $t_{1/2}$ of 103 sec (0.007 sec^{-1}), to a maximal value of approximately 320 fmol/mg.

Because exchange of B_{slow} was not measured explicitly in this series of assays (i.e., B_{slow} was calculated as the portion of total TRH-stimulated binding that was not rapidly exchanged), a portion of B_{slow} is likely to represent radioligand that has become irreversibly bound due to the action of proteases or other unknown processes. However, in numerous extended exchange assays (e.g., see Fig. 4), the fraction of irreversibly bound ligand has never exceeded 25%, whereas for preincubations of 60 sec or more B_{slow} accounted for >50% of the total TRH-stimulated binding. Furthermore, it was demonstrated in Fig. 5 and Table 1 that the accumulation of B_{slow} was specifically reduced when the concentration of TRH was lowered to 17 nM. B_{slow}, as measured in Fig. 6, *inset*, therefore represents primarily an activity that both accumulates and exchanges more slowly than the $G_{q/11}$-related burst, B_{fast}. Importantly, for both B_{fast} and B_{slow}, the binding and exchange processes occurred with similar kinetics, and the rate of nucleotide exchange into B_{fast} and B_{slow} remained essentially constant for the duration of these assays. We conclude that no desensitization of B_{fast} occurs under these experimental conditions.

Comparison of GTP and GTPαS. [^{35}S]GTPαS was chosen for this study because of its relatively mild radioactive decay energy and based on the assumption that the α-thioester linkage would have relatively little impact on its interaction with G proteins. GTP and GTPαS competition assays were performed by incubating membranes with 1 nM [^{35}S]GTPαS plus varying concentrations of either GTP or GTPαS and then measuring bound radioligand after either 3 or 10 min. After 3 min, GTP and GTPαS competed for TRH-stimulated sites with EC_{50} values of 115 and 21 nM, respectively (data not shown). After 10 min, competitive EC_{50} values were 53 and 8 nM, respectively. Thus, GTPαS shows approximately 6-fold greater affinity for the TRH-stimulated sites than does GTP. Higher affinity of GTPαS might be achieved by virtue of a faster association rate, a slower dissociation rate, or the ability of GTPαS to bind to sites inaccessible to GTP. The latter possibility may be ruled out, because high concentrations of GTP reduced [^{35}S]GTPαS binding to background levels (data not shown). Because measurement of dissociation rates faster than those observed for [^{35}S]GTPαS is not possible using the methods of this study, we are presently unable to discriminate between the remaining two possibilities. Thus, the [^{35}S]GTPαS binding rates may overestimate GTP binding rates by up to 6-fold, and the [^{35}S]GTPαS dissociation rate might similarly underestimate the GTP dissociation rate.

Nature of the background [^{35}S]GTPαS binding. Additional characterization of the background binding was performed during efforts to optimize the binding assay. Both the TRH-stimulated binding and the background binding were magnesium dependent over the range of 10 μM to 50 mM, and no improvement in the signal/background ratio was achieved by varying the Mg^{2+} concentration over this range (data not shown). Adenosine, which might be introduced via ATP, inhibits prolactin release by GH_4C_1 cells (31), and adenosine receptors are known to couple to G proteins (32). When GDP was used to reduce the background binding, the ratio of TRH-stimulated binding to background binding was similar to the ratio observed with ATP; however, possible involvement of adenosine receptors in the background binding may warrant further attention. Tubulin is found in plasma membrane preparations from brain (33), and binding of GTP to tubulin might also account for some of the background binding in the GH_4C_1 cell membrane preparations.

To evaluate the 'leakiness' of the TRH-stimulatable sites, i.e., to observe the amount of GTPαS binding to these sites that occurs in the absence of TRH, we incubated membranes with [^{35}S]GTPαS for 5 min in the absence of TRH, added GTP (14 μM) without or with sufficient TRH to achieve a final concentration of 3.5 μM, and then measured the remaining bound GTPαS at 20 sec after chase. In a series of 18 measurements in the absence of TRH we observed 690 $\pm$ 41 fmol/mg (mean $\pm$ standard deviation) bound [^{35}S]GTPαS. When TRH was added to the chase, we observed 665 $\pm$ 37 fmol/mg bound, a small but statistically significant decrease of 25 fmol/mg ($p < 0.05$). We can estimate the degree by which TRH accelerates binding to B_{fast} by comparing the rate in the absence of TRH (25 fmol/mg/300 sec) with the stimulated rate as estimated from Fig. 6 (110 fmol/mg/15 sec). The 90-fold increase in the rate of binding to B_{fast} represents a lower estimate of the rate acceleration induced by TRH, because, although we observed that binding was complete within 15 sec, the time resolution of our methods was insufficient to measure the true rate of binding

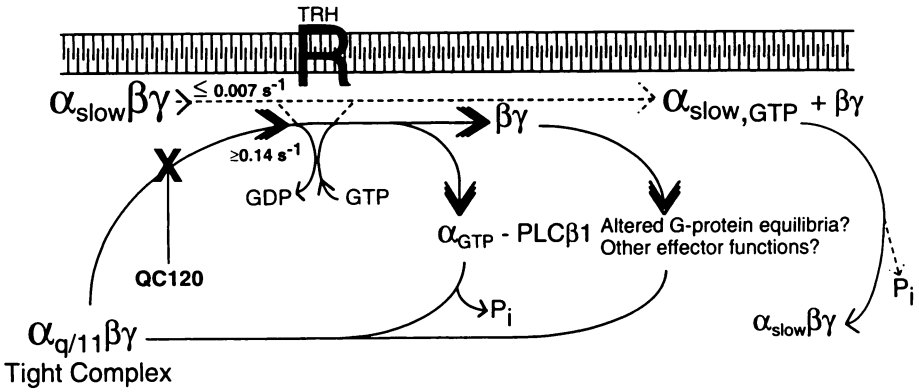

Fig. 7. Proposed kinetics of parallel activation of two G proteins by the TRH receptor. $\alpha_{q/11}\beta\gamma$ and $\alpha_{slow}\beta\gamma$ represent two different heterotrimeric G protein complexes in resting GH_4C_1 cells. The TRH receptor, *R*, catalyzes guanine nucleotide exchange and dissociation of either complex, although its interaction with $\alpha_{slow}\beta\gamma$ is less efficient than that with $\alpha_{q/11}\beta\gamma$ (*dashed lines*). Activation of $\alpha_{q/11}$ is rapid (*triple arrows*). Processes for which rate constants have been estimated in this study are as follows: activation of $\alpha_{q/11}$, $k \geq 0.14$ sec^{-1} (Fig. 6); activation of α_{slow}, $k \leq 0.007$ sec^{-1} (Fig. 6). Because QC120 is able to block both the binding (Fig. 3) and exchange (Fig. 5) of guanine nucleotides to/from $\alpha_{q/11}$, this antiserum acts by blocking the interaction of $\alpha_{q/11}\beta\gamma$ with *R*, as indicated by *X*. $\beta\gamma$ released rapidly after TRH stimulation may be expected to alter the equilibria of other heterotrimeric G proteins and, possibly, to activate specific effector functions (35).

to B_{fast}. We conclude that the coupling of the TRH receptor to its G protein(s) remains tightly regulated in our membrane preparation and leakiness does not contribute substantially to the background binding observed in GH_4C_1 membranes.

Summary of kinetic measurements. Fig. 7 outlines the G protein activation/deactivation cycles that we propose to describe TRH-stimulated events in GH_4C_1 cells. Fig. 7 incorporates demonstrated properties of G_q with observations from the present study. The high affinity of $\alpha_{q/11}$ for $\beta\gamma$ probably contributes to the tight regulation of basal guanine nucleotide exchange by $\alpha_{q/11}$. Furthermore, because activation of $G_{q/11}$ is quite rapid, there would occur a surge of free $\beta\gamma$ after TRH treatment, which might be expected to alter other G protein equilibria in the membranes. The GTP-bound form of $\alpha_{q/11}$ activates phospholipase C-β_1 (8) and becomes activated as a GTPase (11). After hydrolysis of GTP, $\alpha_{q/11}$ transiently recombines with $\beta\gamma$ and re-enters the cycle. Our studies have demonstrated that QC120 blocks both the binding (Fig. 3) and exchange (Fig. 5) of labeled guanine nucleotide to/from $\alpha_{q/11}$, indicating that QC120 blocks interaction of the heterotrimeric G protein with the TRH receptor.

Conclusions. We demonstrated that TRH stimulated binding of the GTP analog GTPαS to GH_4C_1 cell membranes and that this binding occurred on a time scale consistent with the rapid biological responses induced by TRH in these cells (30, 34). Rapid TRH-enhanced GTPαS binding was largely inhibited by an antibody (QC120) prepared against the carboxyl-terminal portion of $\alpha_{q/11}$ (Figs. 2 and 3), consistent with recent observations (8) that a member of this G protein family couples the TRH receptor to phospholipase C-β_1 (11) and consequent rapid generation of diacylglycerol and inositol-1,4,5-trisphosphate. A second guanine nucleotide exchange activity was identified, which was not inhibited by QC120 and which accumulated and exchanged with substantially slower kinetics, compared with the QC120-inhibitable burst. At low agonist concentrations, the accumulation of this activity was greatly reduced, relative to the burst activity, consistent with a mechanism involving parallel activation of two independent G proteins by the TRH receptor. B_{slow} persisted after treatment with pertussis toxin and was fully additive with GTPαS binding stimulated by SRIF, ruling out the involvement of G_o or G_i. The identity and physiological significance of B_{slow} is therefore not yet known.

Acknowledgments

We thank Yolanda Santo, Edward Voelkel, and Joan Joseph for expert assistance in the laboratory and Ms. Jean Foley for the preparation of this manuscript. Purified recombinant $G_{\alpha s}$ was a gift from Alfred Gilman (University of Texas Southwestern Medical Center). Purified antiserum to $G\alpha_s$ was a gift from Patrick Casey (Duke University). Antiserum WO82 was a gift from Paul Sternweiss (University of Texas Southwestern Medical Center).

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