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Dominant Portion of Thyrotropin-Releasing Hormone Receptor Is Excluded from Lipid Domains. Detergent-Resistant and Detergent-Sensitive Pools of TRH Receptor and $G_q\alpha/G_{11}\alpha$ Protein

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Some G protein–coupled receptors might be spacially targetted to discrete domains within the plasma membrane. Here we assessed the localization in membrane domains of the epitope-tagged, fluorescent version of thyrotropin-releasing hormone receptor (VSV-TRH-R-GFP) expressed in HEK293 cells. Our comparison of three different methods of cell fractionation (detergent extraction, alkaline treatment/sonication and mechanical homogenization) indicated that the dominant portion of plasma membrane pool of the receptor was totally solubilized by Triton X-100 and its distribution was similar to that of transmembrane plasma membrane proteins (glycosylated and non-glycosylated forms of CD147, MHCI, CD29, CD44, transmembrane form of CD58, Tapa1 and Na,K-ATPase). As expected, caveolin and GPI-bound proteins CD55, CD59 and GPI-bound form of CD58 were preferentially localized in detergent-resistant membrane domains (DRMs). Trimeric G proteins $G_q\alpha/G_{11}\alpha$, $G_i\alpha1/G_i\alpha2$, $G_s\alpha L/G_s\alpha S$ and Gβ were distributed almost equally between detergent-resistant and detergent-solubilized pools. In contrast, VSV-TRH-R-GFP, Gα, Gβ and caveolin were localized massively only in low-density membrane fragments of plasma membranes, which were generated by alkaline treatment/sonication or by mechanical homogenization of cells. These data indicate that VSV-TRH-R-GFP as well as other transmembrane markers of plasma membranes are excluded from TX-100–resistant, caveolin-enriched membrane domains. Trimeric G protein $G_q\alpha/G_{11}\alpha$ occurs in both DRMs and in the bulk of plasma membranes, which is totally solubilized by TX-100.

Key words: $G_q\alpha/G_{11}\alpha$, lipid domains, thyrotropin-releasing hormone receptor, TRH, trimeric G proteins.

Abbreviations: AC, adenylyl cyclase; BPB, bromphenol blue; DRMs, detergent-resistant or detergent-insensitive membrane domains; DMEM, Dulbecco's modified Eagle's medium; G proteins, heterotrimeric guanine nucleotide–binding regulatory proteins; $G_s\alpha$, G protein stimulating adenylyl cyclase activity; $G_i\alpha1/G_i\alpha2$, G proteins inhibiting adenylyl cyclase activity in pertussis-toxin sensitive manner; $G_q\alpha/G_{11}\alpha$, G proteins stimulating phoshoplipase C in pertussis-toxin independent manner; GPCR, G protein coupled receptor; GFP, green fluorescent protein; HEK, human embryonic kidney; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline; PMs, plasma membranes; PMSF, phenylmethylsulfonyl fluoride; Na,K-ATPase, sodium plus potassium activated, ouabain-sensitive adenosine triphosphatase [EC 3.6.1.3]; SLB, solubilization lysis buffer; TBS, Tris-buffered saline, VSV, vesicular stomatitis virus; TRH, thyrotropin-releasing hormone; TRH-R; thyrotropin-releasing hormone receptor; VSV-TRH-R-GFP, N-terminally VSV-tagged form of TRH-R-GFP fusion protein.

Plasma membranes (PMs) of hormone-responsive cells can no longer be viewed as a homogeneous mixture of freely mobile GPCRs and G proteins as was originally assumed by the collisional coupling hypothesis (*1–6*). Membrane domains (also known as detergent-resistant membrane fragments (DRMs), detergent-insensitive/insoluble membrane fragments (DIMs), sphingolipid-cholesterol membrane rafts, glycolipid-enriched membranes (GEMs), detergent-insoluble glycolipid-enriched domains (DIGs) or caveolae), have been suggested to be intimately involved in membrane signal transduction (*7–10*). To date, however, the quantitative proportion between GPCRs and trimeric G proteins in various subcellular membrane fractions is not well known because all previous efforts to estimate the G protein/GPCR stoichiometry were oriented just to analyzing the whole cell or crude membrane preparations (*11–16*). It is also not clear which portion of totally plasma membrane-bound GPCRs or G proteins is present in DRMs.

Therefore, in this work, HEK293 cells stably expressing the epitope-tagged, fluorescent version of thyrotropin-releasing hormone receptor (VSV-TRH-R-GFP) were utilized as an advantageous methodological tool to assess the receptor distribution by radioligand binding, fluores-

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cence and immunoblot analyses. These parallel assays allowed detection of the receptor protein in particulate membrane as well as detergent-solubilized fractions. The immunoblot data were corroborated by fluorescence analysis and *vice versa*. The distribution of VSV-TRH-R-GFP in low-density, detergent-resistant membrane domains (DRMs) and in the detergent-solubilized pool was compared with the distribution of trimeric G proteins, caveolin and other plasma membrane constituents. Cell membrane fragments were prepared by a simple mechanical disintegration of intact cells (*i.e.*, homogenization). This approach enabled us to compare our immunoblot and fluorescence data with a direct quantitative assay (radioligand binding) of the receptors. We also studied "detergent-free" membrane preparations obtained by alkaline treatment (0.5 M $NaHCO_3$, pH 11) and sonication. The distribution of selected membrane markers representing integral (transmembrane) or peripheral GPI-bound proteins was analyzed in parallel.

MATERIALS AND METHODS

Chemicals—Tissue culture reagents and media were supplied by Sigma or Gibco. All other chemicals and drugs, including geneticin and hygromycin, were purchased from Sigma (St. Louis, MO, USA). Antibodies against caveolin (C136300), caveolin-2 (C57820) and flotillin-2/ESA (E35820) were purchased from Transduction Laboratories (Nottingham, U.K.) and antibodies against $G_s\alpha$ (G-5040) and VSV (V-5507) were from Sigma. Anti–VSV-G monoclonal antibody (1667351) was from Roche Diagnostic (Mannheim, Germany) and anti-GFPpAb (sc-8334) and anti-Gβ (sc-378) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [α-^{32}P]ATP (30 Ci/mmol; PB 171), [^{3}H]CGP12177 (46 Ci/mmol, TRK 835), [21,22–^{3}H]ouabain (32 Ci/mmol; TRK 429) and [^{3}H]dihydroalprenolol (40 Ci/mmol; TRK 839) were purchased from Amersham-Pharmacia Biotech. (Buckinghamshire, UK). Aluminium oxide 90 (neutral, activity I, cat. no. 101077-2000) was from Merck (Darmstadt, Germany). Complete protease inhibitor cocktail was from Roche Diagnostic (Mannheim, Germany). [^{3}H]TRH was prepared by Drs. Hlavacek and Cerny from the Institutes of Organic Chemistry and Plant Physiology, Academy of Sciences of the Czech Republic (Prague, CZ).

Cell Culture—Parental or transfected clones of HEK293 cells stably expressing VSV-epitope tagged version of TRH-R-GFP fusion protein (*17*) were cultivated in minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% new born calf serum at 37°C. Geneticin (800 μg) was included in the course of cell cultivation. Cells were grown to 60–80% confluency before harvesting and starting of experiments.

Isolation of Plasma Membranes by Flotation in Sucrose Density Gradients. Subcellular Fractionation after Mechanical Homogenization—HEK293 cells expressing VSV-TRH-R-GFP were harvested from 6 flasks (80 cm^2 each). Cells were collected by low-speed centrifugation at 1,800 rpm for 10 min, washed once in PBS and resuspended (on ice) by repeated pipetting in 1 ml of 250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA. Cell suspension was diluted to the same protein concentration in control and agonist-stimulated samples, fresh PMSF (200 mM stock in isopropyl alcohol) was added to a final concentration of 1 mM PMSF along with a complete protease inhibitors mixture, and the final volume was adjusted to 2 ml. Immediately afterwards, mechanical homogenization was carried out for 7 min at 1,800 rpm in a tightly fitting, teflon-glass homogenizer (Brown, Germany). Precisely 2 ml of the resulting homogenate (15–20 mg protein/ml in various isolation procedures) was mixed with 2 ml of ice-cold 80% w/v sucrose, transferred into a centrifuge tube of Beckman SW 41 rotor and overlaid with 35, 30, 25, 20, 15, 10 (1 ml each) and 5% w/v sucrose (1.5 ml). Great care was taken to prevent the lower and higher sucrose layers from mixing during preparation of this gradient. Sucrose density gradient fractions 1 (0.5 ml, 5%), 2 (1 ml, 5%), 3 (1 ml, 10 %), 4 (1 ml, 15%), 5 (1 ml, 20%), 6 (1 ml, 25%), 7 (1 ml, 30%), 8 (1 ml, 35% sucrose) and 9–12 (1 ml, 40% sucrose) were collected manually from the meniscus after centrifugation for 24 h at 39,000 rpm (187,000 × *g*).

Subcellular Fractionation after Detergent Extraction—Separation of DRMs from Detergent-Solubilized Cell Constituents—In most of the previous studies of membrane domain/raft compartments (*18–22*), only a simple "5/35/40" type of sucrose density gradient was used for isolation of DRMs. DRMs were collected from the 5/35% (w/v) sucrose interphase. This type of density gradient, however, does not allow for separation of various forms of DRMs differing in their buoyant density. By using a more complicated step-wise "5/10/15/20/25/30/35/40" type of gradient, we were able to demonstrate that the distribution of $G_q\alpha/G_{11}\alpha$ protein clearly differed from that of caveolin. This could not be achieved with a "5/35/40" sucrose gradient (*23*). We also observed that 1% TX-100 extraction or high-energy ultrasound exposure (sonication) abolished the functional coupling between a GPCR and its cognate G protein assayed as an agonist-stimulated GTPγS binding (*23*). Agonist stimulation of adenylyl cyclase was diminished as well (*23*). Therefore, in our present study, a "5/10/15/20/25/30/35/40" type of sucrose gradient was used regularly, and simple mechanical homogenization and detergent extraction were compared as means of degradation of intact cell structure. Besides these two procedures, the alkaline treatment protocol introduced by Song *et al.* (*24*, *25*) was also used for isolation of detergent-free preparations of membrane domains. When using this procedure, however, we applied only a mild sonication under carefully controled conditions (at 0–4°C).

HEK293 cells were harvested from 6 flasks (80 cm^2 each), collected by centrifugation at 1800 rpm for 10 min (0–4°C), washed once in PBS and resuspended on ice by repeated pipetting in 1 ml of 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$ and 1 mM EDTA (TME buffer). Cell suspension was diluted to the same protein concentration in control and agonist-stimulated samples, and fresh PMSF (200 mM stock in isopropyl alcohol) was added to a final concentration of 1 mM PMSF along with a complete protease inhibitors mixture. Final volume of cell suspension was adjusted to 1 ml. Subsequently, 1 ml of ice-cold 2% (v/v) TX-100 in TME buffer plus PMSF was added and slowly mixed with the cell suspension by repeated pipetting. The ratio between the detergent (TX-100) and homogenate protein was between 0.4:1 and 0.6:1 in dif-

ferent isolation procedures. After 60 min on ice (box with ice in a cold room equilibrated to 0–4°C), 2 ml of 1% TX-100-cell extract was mixed with 2 ml of ice-cold 80% (w/v) sucrose, transferred into a centrifuge tube of a Beckman SW 41 rotor and overlaid with 35, 30, 25, 20, 15, 10 (1 ml each) and 5% w/v sucrose (1.5 ml) as described before. Gradient fractions 1–12 (1 ml each) were collected manually from the meniscus after centrifugation for 24 h at 39,000 rpm (187,000 × *g*).

Subcellular Fractionation after Alkaline Treatment/Sonication—This type of membrane domain preparation was adapted from the procedure of Song *et al.* (*24*, *25*). HEK293 cells were collected from 6 flasks (80 cm^2 each) by a low-speed centrifugation (1,800 rpm, 10 min, 0°C), washed once in PBS and diluted with TME buffer containing 1 mM PMSF and protease inhibitors to the same protein concentration in control and TRH-treated samples. The resulting cell suspension was adjusted to a volume of 1 ml, mixed with 1 ml of ice-cold 1 M Na_2CO_2 (pH 11), sonicated three times for 10 s in an ice-water slurry at low energy output (*26*) and transferred into a centrifuge tube of a Beckman SW 41 rotor. This homogenate was then mixed with 2 ml of ice-cold 80% w/v sucrose, overlaid with 35, 30, 25, 20, 15, 10 (1 ml each) and 5% (1.5 ml) sucrose and centrifuged for 24 h at 39,000 rpm (187,000 × *g*). Density gradient fractions 1–12 were collected from top to bottom as described above.

Production of Antisera—G-protein selective antibodies were polyclonal antipeptide sera raised in rabbits. Immunization was performed with a glutaraldehyde conjugate of keyhole limpet hemocyanin and synthetic peptides specific for a given type of G protein. Peptide QLNLKEYNLV representing the C-terminal decapeptide, which is conserved between $G_q\alpha$ and $G_{11}\alpha$, was used for preparation of CQ antiserum. This antiserum identifies both polypeptides equally. Antiserum SG was raised against a peptide corresponding to the C-terminal decapeptide of transducin, KENLKDCGLF (amino acids 341–350). Antiserum CS was raised against a peptide corresponding to the C-terminal decapeptide of $G_s\alpha$, RMHLRQYELL (amino acids 385–394).

Immunoblot Analysis of Sucrose Density Gradient Fractions—Sucrose density gradient fractions (constant volume of 0.75 ml) were precipitated with trichloroacetic acid (0.25 ml, 6% w/v, 1 h on ice), resuspended in 20 μl of 1 M Tris-base and solubilized by addition of 20 μl of 2× concentrated Laemmli buffer with or without urea (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.4 M DTT, 0.04% BTB). The samples were heated for 3 min at 95°C. In some cases, density gradient fractions were also analyzed using constant protein (20 μg/sample). Standard SDS-PAGE in 10% w/v acrylamide/0.26% w/v bisacrylamide or 12.5% w/v acrylamide/0.0625% w/v bisacrylamide gels was carried out as described (*27*). Molecular mass determinations were based on prestained molecular mass markers (Sigma, SDS 7B).

After SDS-PAGE, proteins were transferred to nitrocellulose and blocked for 1 h at room temperature in 3% (w/v) low-fat milk in TBS-T buffer [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20]. The G-protein–specific antisera were added in TBS-T buffer containing 1% (w/v) low-fat milk and incubated for at least 2 h. The primary antibody was then removed and the blot washed extensively (3 × 10 min) in TBS-T buffer. Secondary antibodies (donkey anti–rabbit IgG or sheep anti–mouse IgG conjugated with horse-radish peroxidase) diluted in TBS-T buffer containing 1% (w/v) low-fat milk were applied for 1 h, and after three 10-min washes the blots were developed by ECL technique using Super Signal West Dura (Pierce) as substrate. The developed blots were scanned with an imaging densitometer Astra 610P (UMAX) to enable quantification of the immunoblots.

Measurement of GFP Fluorescence—Endogenous GFP fluorescence of VSV-TRH-R-GFP protein was measured in 0.1 ml aliquots of sucrose density fractions 1–12 using Wallac Victor spectrofluorometer in fluorescein isothiocyanate mode (485/520 nm).

Detection of Membrane Marker Molecules—Plasma membrane proteins CD29 (integrin β1 subunit) (*20*), CD44 (lymphocyte homing receptor–phagocytic glycoprotein) (*28*), MHCI (major histocompatibility complex, class I), transmembrane form of CD58 (formerly called LFA-3) (*29*) and glycosylated and non-glycosylated forms of CD147 (M6) (*30*) were measured as markers of transmembrane, integral membrane proteins. CD55 (complement decay accelerating factor) (*31*), GPI-bound form of CD58 (LFA-3) (*29*) and CD59 (complement protectin) were measured as markers of GPI-bound, peripheral proteins (for comprehensive information, see Ref. *32*). Characteristic behavior of these proteins in the course of detergent extraction and separation on equilibrium sucrose density gradients has been established in a number of previous studies performed in T lymphocytes and monocytes (*1*, *9*, *33–36*). All CD membrane markers were detected by immunoblotting with monoclonal antibodies obtained as a kind gift from the collection of Prof. V. Horejsi (Institute of Molecular Genetics, Prague, CZ); the immunoblot signals were detected by ECL technique according to Nesbitt and Horton (*37*). As a classical marker of integral membrane proteins, sodium plus potassium-activated, magnesium-dependent adenosine triphosphatase [EC 3.6.1.3] (Na,K-ATPase) was detected by antibodies purified by protein A-affinity chromatography from rabbit polyclonal antiserum, which was prepared against the isolated α1-subunit of this enzyme (kind gift of Prof. Roberto Antolovic, Giessen University, Germany).

Adenylyl Cyclase—Adenylyl cyclase enzyme activity (*38*) was determined in aliquots of density gradient fractions, which were snap-frozen in liquid nitrogen and thawed only once. A constant volume (40 μl) of each sample was incubated for 30 min at 30°C in a total volume of 0.1 ml of 70 mM HEPES/HCl (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EDTA, 16 mg/ml pyruvate kinase, 10 mM potassium phosphoenolpyruvate, 160 mg/ml BSA, 0.1 mM ascorbate, 0.01 mM RO-201724 (phosphodiesterase inhibitor) and 0.15 mM ATP plus [^{32}P]ATP (about 1×10^6 cpm per sample). The activating ligands (2.10^{-5} M GTP; 1.10^{-5} M isoprenaline) were added 5 min before starting the enzyme reaction by addition of ATP plus [^{32}P]ATP. The enzyme reaction was terminated by addition of 0.1 ml of 0.25% SDS/5 mM ATP/0.175 mM cyclic AMP plus cyclic [^{3}H]AMP (20,000 cpm per sample as an internal standard) and heating for 5 min at 95°C. Cyclic AMP was separated from other nucleotides and inorganic phos-

phate by alumina column chromatography as described by White (*39*).

Radioligand Binding Assays—TRH receptors: Distribution of TRH receptors was determined by "one-point" radioligand binding assay using 10 nM [^{3}H]TRH. Constant volumes (100 μl) of density gradient fractions were incubated for 30 min at 30°C in a total volume of 0.5 ml of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA. The bound and free radioactivity was separated by filtration through Whatman GFB filters on Brandel Cell harvestor, and non-specific binding was measured in the presence of 10 μM unlabeled TRH.

Na,K-ATPase: Distribution of Na,K-ATPase along the density gradient was determined by [^{3}H]ouabain binding assay as described by Svoboda *et al.* (*40*). Portions of density gradient fractions (100 μl) were incubated with [^{3}H]ouabain in a total volume of 0.5 ml of 5 mM NaH_2PO_4, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.6) at 37°C. After 5–7 min, the binding reaction was initiated by addition of [^{3}H]ouabain (5 nM final concentration) and continued for 90 min. Incubations were terminated by dilution with 5 ml of ice-cold incubation buffer and filtration through Whatman GFB filters that had been impregnated in 0.3% polyethyleneimine for 1 h at laboratory temperature. The filters were washed twice with 5 ml of cold buffer, dried overnight at laboratory temperature, and radioactivity was determined by liquid scintillation in 4 ml of CytoScint (ICN). Non-specific binding was determined in the presence of 1 μM unlabeled ouabain.

β-Adrenergic receptors: β-Adrenergic receptor content was determined by radioligand binding using the hydrophilic antagonist [^{3}H]CGP12177. The radioligand was applied at 10 nM concentration for assessment of the density gradient profile or in the range of 0.15–20 nM for construction of saturation binding curves and subsequent determination of B_{max} (maximum number of binding sites) and K_d values.

RESULTS

Separation of Low-Density Membrane Fragments from the Bulk Phase of Plasma Membranes by Flotation in Sucrose Density Gradients—A routine way to isolate detergent-resistant membrane domains (DRMs) is by flotation in sucrose density gradients. The detergent extract is applied to a centrifuge tube and mixed with the same volume of highly concentrated (80%) sucrose solution. This mixture (containing 40% sucrose) is then overlaid with sucrose layers of lower densities and DRMs float up to the regions of low density (about 15–20% sucrose) in the course of long-term high-speed centrifugation. The detergent-solubilized material including soluble proteins does not float and remains in the bottom 40% sucrose layer. Cell constituents exhibiting higher buoyant density than that corresponding to 40% sucrose sediment down to the gradient pellet.

Current methods for isolation of DRMs do not allow comparison of the "low-density, detergent-resistant material" (*i.e.*, *domains*) with the detergent-untreated plasma membrane fraction. In order to compare directly these two types of membrane structures, analogical techniques need to be applied for preparation of both DRMs and plasma membranes (PMs). Since DRMs are exclusively isolated by flotation in density gradients, representative preparations of PMs should be also obtained by using the flotation mode of density gradient centrifugation.

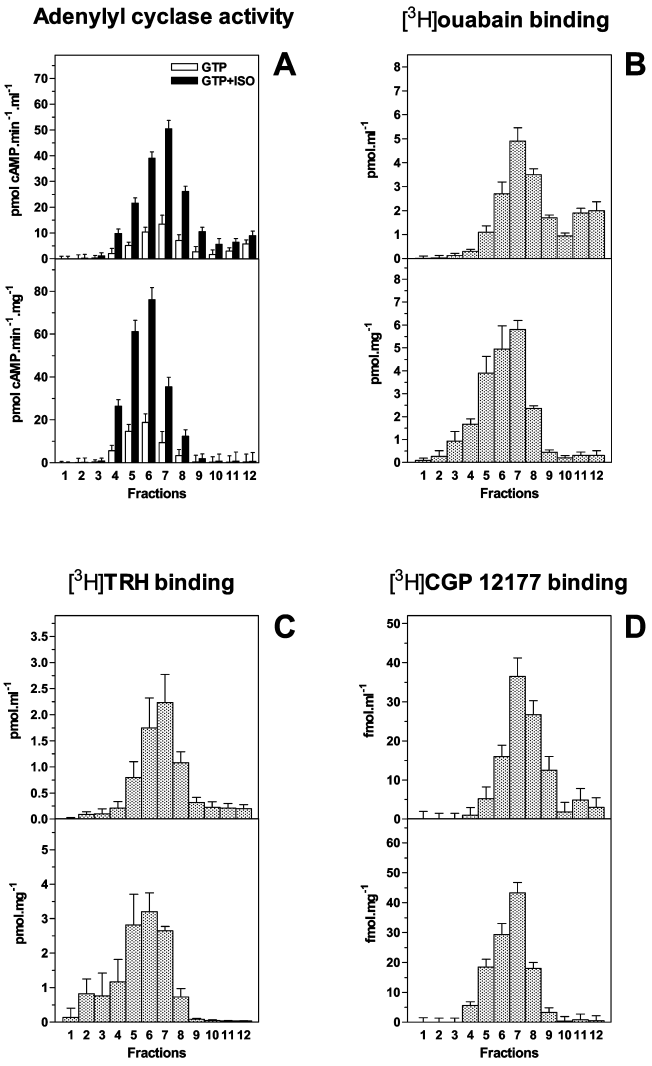

Fig. 1. **Distribution of plasma membrane markers along the flotation density gradient.** Fractions were collected from the step-wise 5/10/15/20/25/30/35/40% sucrose density gradient and analyzed for GTP- or GTP/isoprenaline-stimulated adenylyl cyclase (A), [^{3}H]ouabain binding (B), [^{3}H]TRH binding (C) and [^{3}H]CGP12177 binding (D) as described in "METHODS AND MATERIALS." Results are expressed as enzyme activity or specific binding capacity per fraction and per mg protein. Five independent fractionation procedures were performed to collect all the data, and each gradient profile represents a single typical fractionation procedure analyzed in triplicate.

Here we isolated PMs from HEK293 cells expressing the VSV-epitope–tagged, fluorescent version of thyrotropin-releasing hormone receptor (VSV-TRH-R-GFP) by flotation in an equilibrium sucrose density gradient consisting from 5/10/15/20/25/30/35 and 40% (w/v) sucrose. When performed under optimum conditions, there was no major difference between the *flotation* and *sedimentation* modes of subcellular fractionation. In "flotation" gra-

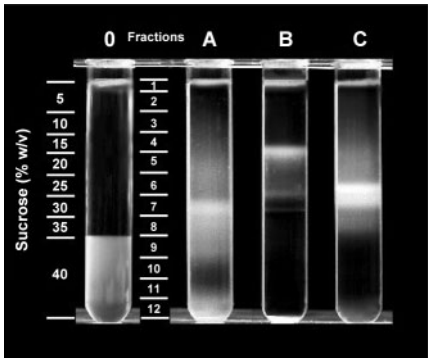

Fig. 2. **Macroscopical picture of the density gradients.** HEK293 cells obtained from 18 flasks (80 cm^2 each) were harvested by low-speed centrifugation and suspended in 3.5 ml of TME buffer, and 1 ml of this suspension was used as starting material for fractionation according to the homogenization (A), detergent extraction (B) or alkaline-treatment/sonication (C) protocols (see "MATERIALS AND METHODS"). In this way, the same amount of homogenate protein (18 mg) was applied to each gradient. The photographs of density gradient profiles were taken before (O) and after centrifugation (A, B, C) for 24 h at 187,000 × *g* (39,000 rpm) in a Beckman SW41 rotor.

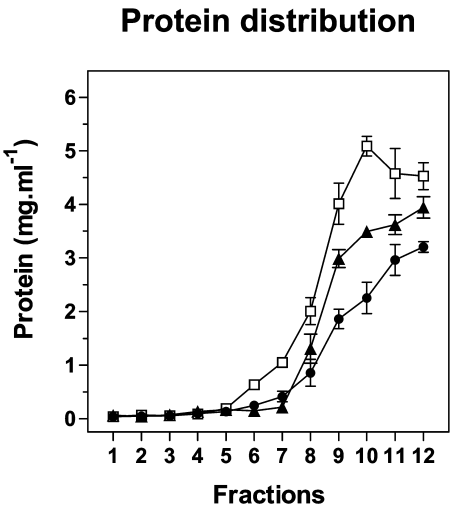

Fig. 3. **Protein distribution in the density gradients.** Protein concentration was determined in the individual fractions obtained from density gradients prepared according to the homogenization (solid circles), detergent extraction (solid triangles) or alkaline treatment/sonication (open squares) protocols. The total amount of protein applied on each gradient was 22 mg. About 45% (homogenization), 39% (TX-100 extraction) and 7% (alkaline treatment/sonication) of protein remained in the gradient pellet. Data shown are the means from three independent fractionation experiments measured in duplicate.

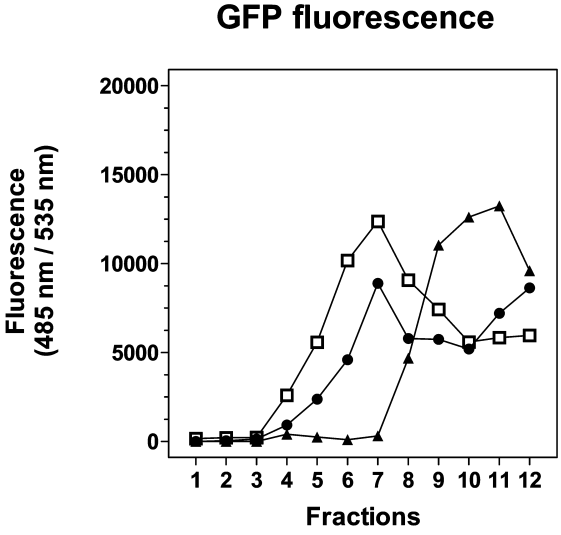

Fig. 4. **Detection of VSV-TRH-R-GFP protein by measurement of GFP fluorescence.** Identical amounts of HEK293 cells (each corresponding to 6 × 80 cm^2) expressing VSV-TRH-R-GFP fusion protein were fractionated according to the homogenization (solid circles), detergent extraction (solid triangles) or alkaline treatment/sonication (open squares) protocols (see "MATERIALS AND METHODS"). GFP fluorescence (E_x 485 nm/E_m 535 nm) was determined in 100-μl aliquots of all gradient fractions.

dients, the bulk of PMs was localized in 30–35% (w/v) sucrose. This is the same range of sucrose concentrations where plasma membrane fragments were localized in our previous studies (*41–43*). In those studies, cell homogenates were fractionated by rate-zonal sedimentation down through the density gradient.

The highest activity of adenylyl cyclase and the highest levels of specific binding of [^{3}H]ouabain (Na,K-ATPase), [^{3}H]TRH (TRH receptors) and [^{3}H]CGP12177 (β-adrenergic receptors) were detected in fractions 6–8 (Fig. 1, A–D). These fractions corresponded to 30–35% sucrose. In some experiments, this band was also localized in the 35–40% sucrose interphase or in the top layer of 40% sucrose. These variances are explicable by small differences in experimental conditions (homogenization, mixing of sucrose solutions or temperature of sucrose layers) in the individual fractionation procedures. To the naked eye PMs were visible as a dense whitish band in the 30–35% (w/v) sucrose area, as can be seen in the photographs of density gradients obtained after 24 h of centrifugation at 39,000 rpm (187,000 × *g*) in a Beckman SW41 rotor (Fig. 2). The protein distribution in the individual fractions of density gradients prepared after mechanical homogenization, TX-100 extraction or alkaline treatment/sonication of cells is shown in Fig. 3.

TRH Receptor, Gα, Gβ and Caveolin Are Co-localized in Bulk of Plasma Membranes—Results of [^{3}H]TRH radioligand binding assay presented in Fig. 1C corresponded well to the distribution of VSV-TRH-R-GFP receptor protein obtained by measurement of GFP fluorescence (Fig. 4). The peak of GFP fluorescence was detected in fraction 7. Accordingly, the dominant portion of $G_q\alpha/G_{11}\alpha$, $G_i\alpha1/G_i\alpha2$ and Gβ subunit proteins (Fig. 5, A–C, upper columns) was localized in fractions 6–8. The same was true for Na,K-ATPase measured as [^{3}H]ouabain binding (Fig. 6) and isoprenaline (β-adrenergic receptor agonist)-stimulated adenylyl cyclase, which exhibited the highest activity in fraction 7 (Fig. 7A). The assay of isoprenaline-stimulated adenyl cyclase indicated that functional coupling between endogenously expressed β-adrenergic receptor, trimeric G protein $G_s\alpha$ and the effector molecule of adenylyl cyclase was preserved in these plasma membrane fragments. It might be concluded, therefore, that fractions 6–8 contain the bulk of PM with undiminished

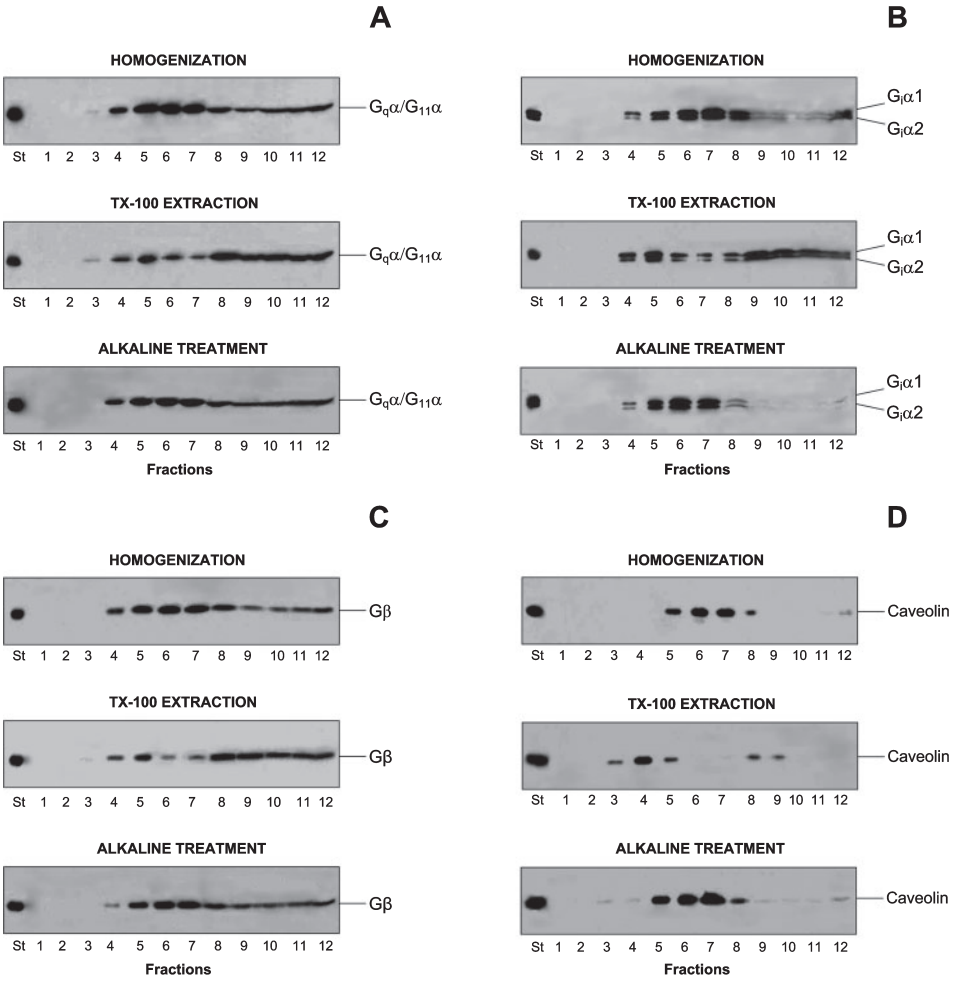

Fig. 5. **Distribution of trimeric G proteins and caveolin in the density gradient.** HEK293 cells obtained from 18 flasks (80 cm^2 each) were collected by low-speed centrifugation and suspended in 3.5 ml of TME buffer, and 1 ml of this suspension was used as starting material for fractionation according to the homogenization (upper columns), detergent extraction (middle columns) or alkaline treatment/sonication (lower columns) protocols (see "MATERIALS AND METHODS"). The same amount of homogenate protein (22 mg) was applied to each gradient. Protein concentration was determined in 100-μl aliquots of all gradient fractions; blank values were obtained from 100-μl aliquots of fractions collected from parallel gradients prepared without cell homogenates. Constant volumes (20 μl) were analyzed by SDS-PAGE and immunoblotted for $G_q/G_{11}\alpha$ (A), $G_i\alpha1/G_i\alpha2$ (B), Gβ (C) and caveolin (D). St, rat brain microsomes, 20 μg.

coupling efficiency between crucial components of the β-adrenergic signaling system of adenylyl cyclase.

Very similar distribution was obtained for caveolin (caveolin-2), a typical marker of DRMs, which also exhibited the highest immunoblot signal in fractions 6–8 (Fig. 5D, upper column). Thus, under standard conditions of cell fractionation (*i.e.*, those using simple mechanical degradation of cell structure), TRH-R, G proteins and caveolin were co-localized in the bulk of PMs.

Separation of Low-Density Membrane Fragments (Light Vesicles) from the Bulk of Plasma Membranes—In the macroscopic picture of density gradient obtained from mechanically homogenized cells (Fig. 2A), the low-density membrane fragments or light vesicles [the term used in our previous studies (*27*, *42*, *43*)], were visible as a hazy area in 15–20% (w/v) sucrose. The content of GPCRs, G proteins and caveolin in this fraction was low in comparison with the bulk of PMs (Fig. 1). Low-density membrane fragments prepared by homogenization represent heterogeneous mixture of vesicular structures of different size, shape and origin and, in hormone pretreated cells, they contain the internalized forms of GPCRs and G proteins released from the bulk phase of PMs by prolonged agonist stimulation (*27*, *42*, *44*, *45*).

The Bulk of Plasma Membranes Is Solubilized by Triton X-100—Detergent extraction (Triton X-100, 60 min at 0°C) exerted a dramatic effect on the density gradient profile, which is clearly visible on the macroscopic photograph of a centrifugation tube after gradient centrifugation (compare Fig. 2, A and B). The distinct band of optically dense material containing plasma membrane fragments after mechanical cell destruction (Fig. 2A) was completely removed in the case of detergent solubilization (Fig. 2B). The disappearance of the plasma membrane band was reflected by marked change in the density gradient profile of TRH receptor protein (Fig. 3). The GFP-fluorescence signal in fractions 6–7 vanished, and the peak of fluorescence intensity was transferred to the non-floating, detergent-solubilized area in 40% sucrose (high-density fractions 9–12). Parallel assay of TRH-R by [^{3}H]TRH radioligand binding indicated solubilization of TRH-R (Fig. 8). There was almost no radioactivity remaining on Whatman GFB, GFF, GFC (fibre glass) or Millipore (0.2 μm) filters in fractions 6–12; very low binding was detected in low-density fraction 2–5 (Fig. 8B). The same was true for Na,K-ATPase and β-adrenergic receptors estimated as [^{3}H]ouabain (Fig. 6) or [^{3}H]CGP12177 binding sites (data not shown). TX-100 extraction exerted an equally drastic effect on receptor coupling efficiency to the downstream effectors. Agonist (isoprenaline)-stimulated adenylyl cyclase was completely diminished, and only the basal level of activity remained (Fig. 7B).

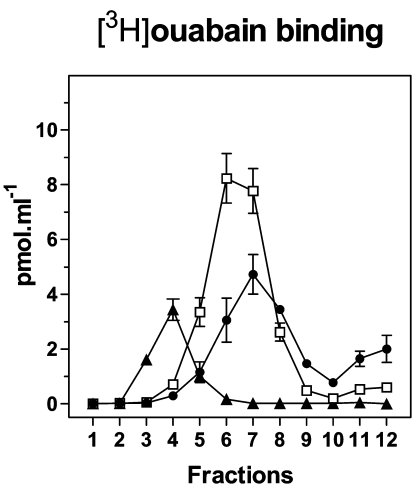

Fig. 6. **[3H]ouabain binding (Na,K-ATPase).** Specific [3H]ouabain binding sites were determined in 25-μl aliquots of gradient fractions at 20 nM total concentration of this radioligand (see "MATERIALS AND METHODS"). Solid circles, homogenization; solid triangles, detergent extraction; open squares, alkaline treatment/sonication. Results shown represent the mean of three fractionation procedures each analyzed in triplicate.

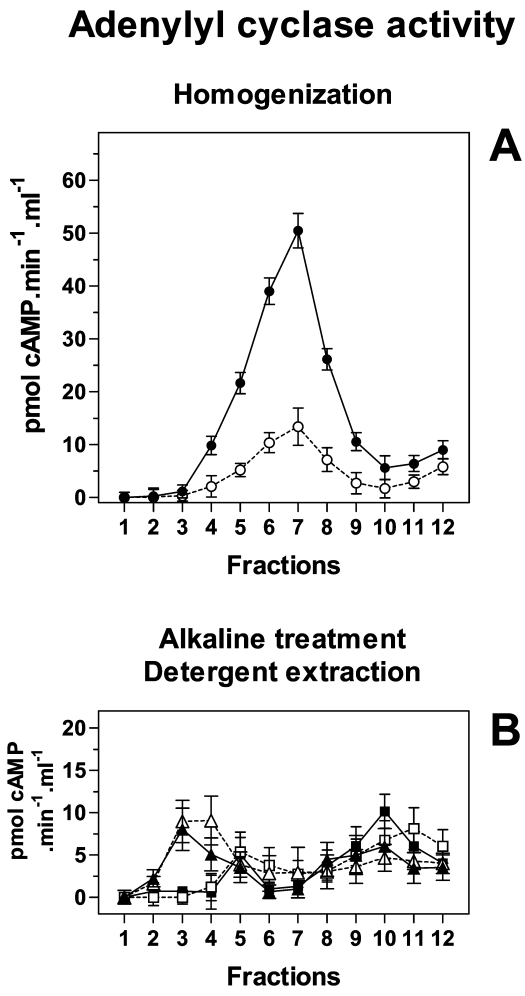

Fig. 7. **GTP- and isoprenaline-stimulated adenylyl cyclase.** Gradient fractions were prepared by homogenization (circles), detergent extraction (triangles) or alkaline-treatment/sonication (squares) protocols and assayed for GTP- (open symbols) or GTP + isoprenaline-stimulated (closed symbols) adenylyl cyclase activity. Results shown are representative of a single fractionation procedure analyzed in triplicate.

Gα and Gβ Subunits in DRMs—Screening of constant-volume aliquots of density gradients for Gα and Gβ subunits indicated that about 20% of the total amount applied per gradient was recovered in DRMs (low-density area of 15–20% sucrose); a much larger portion (about 80%) was detected in fractions 9–12. The latter fractions contained the detergent-solubilized pool of proteins (Fig. 5, A–C, middle columns). The ability of Gβ subunits to float was lower than that of Gα subunits. Sensitivity of Gα proteins to detergent extraction was thus intermediate between TRH-R, which was totally solubilized and remained in the high-density area, and caveolin, which was predominantly localized in DRMs (Fig. 5D, middle column).

The Constant-Volume Mode of Screening the Density Gradients Is the Only Way to Determine the Relative Proportion of Caveolin and G Proteins in DRMs—The current literature mostly does not specify in sufficient detail methods to screen density gradients for signaling molecules such as caveolin, G proteins or GPCRs. This lack of information is easily noticeable if one asks simple questions like: "*What is the recovery of G proteins or caveolin in DRMs when compared with detergent-solubilized pools of these molecules?*" or "*What is the percentage of G proteins or caveolin in DRMs when compared with total amounts of these proteins present in the cell?*" Our results shown in Fig. 9 indicate that the relative amounts of G_q/$G_{11}\alpha$, $G_s\alpha$, caveolin and flotillin in DRMs depend on the way the density gradients are screened. When the same amount of protein is applied from each fraction, the content of Gα subunits in the high-density area (detergent-solubilized pool) is largely underestimated (Fig. 9, upper columns). The same is true for caveolin and flotillin. This is due to an almost two-order difference in protein content between the low- and high-density fractions (Fig. 3). A more appropriate way to determine the ratio of a given protein molecule in DRMs and in the detergent-solubilized pool is to compare the *constant-volume* aliquots taken from each gradient fraction (Fig. 9, lower columns). Under these conditions, the relative amount of relevant proteins in the non-floating, detergent-solubilized pool could have been directly compared with that in DRMs. Importantly, these methodological remarks are not as trivial as they may seem because the majority of current literature data does not specify how the density gradient profiles were analyzed. It is obvious that the *constant-protein* mode of screening of density gradients results in an *artificial increase*, *i.e.*, in overestimation, of the relative G protein content in DRMs.

Majority of VSV-TRH-R-GFP Protein Is Excluded from DRMs—As demonstrated in Fig. 4 (GFP fluorescence) and Fig. 8 ([3H]TRH binding), the dominant portion of VSV-TRH-R-GFP protein originally associated with PMs was solubilized with 1% TX-100 and not retained on Whatman or Millipore filters; only a very low level of [3H]TRH binding was detectable in DRMs. These observations are in line with the notion that TX-100 may affect

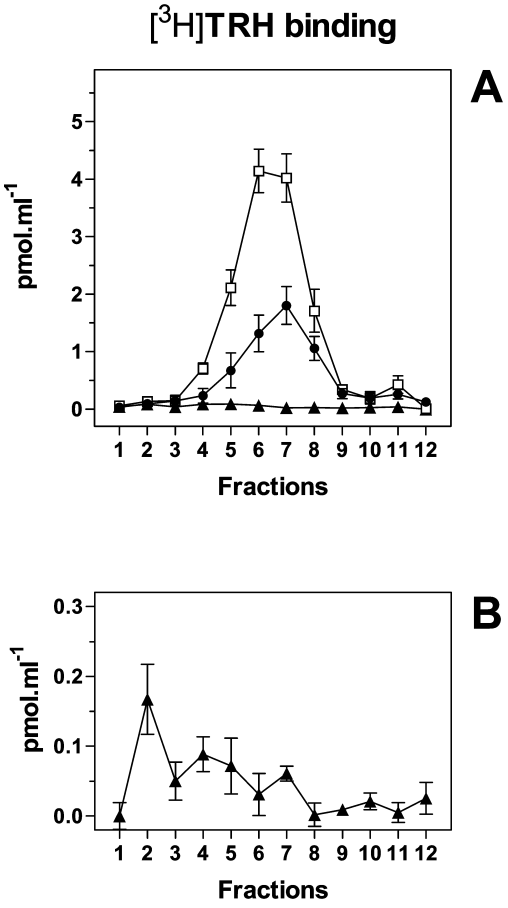

Fig. 8. **[^{3}H]TRH binding.** The density gradient profiles of receptor protein were determined by measurement of the number of specific [^{3}H]TRH binding sites in 100-μl aliquots of density gradient fractions using 10 nM total concentration of the radioligand (A). Non-specific binding was determined in the presence of 1 μM unlabeled TRH. Results shown represent the mean of three independent fractionation procedures analyzed in triplicate. In detergent-treated samples, the same radioligand binding experiment was carried out with 40 nM [^{3}H]TRH (B). Solid circles, homogenization; solid triangles, detergent extraction; open squares, alkaline treatment/sonication.

the conformation and binding activity of receptors (*46*, *47*), but it does not apparently distort the fused GFP conformation. Receptor distribution was further characterized by immunoblotting analyses. The non-floating, detergent-solubilized pool of VSV-TRH-R-GFP in the high-density area was quantified by immunoblotting with anti-GFP antibody as well as by measurement of GFP fluorescence, and compared with the receptor amount determined in DRMs (Fig. 10). The ratio between these two pools was roughly 1:30. About 1–3% of VSV-TRH-R-GFP originally present in PMs was detergent-resistant and transferred to DRMs; the remaining major part was solubilized and occurred in non-floating, high-density fractions 9–12. The distribution of VSV-TRH-R-GFP was also determined using anti-VSV antibody (Fig. 11). Results of these experiments demonstrated that only a small part of the total receptor signal remained in the gradient pellets: about 18% (homogenization), 3% (detergent extraction) or 1% (alkaline treatment/sonication). These observations indicate that TX-100 solubilized primarily the bulk phase of PMs containing the vast majority of VSV-TRH-R-GFP and that the partial solubilization of the pellet could only slightly contribute to the strong receptor signal determined in high-density fractions.

The difference in sensitivity of TRH-R, G proteins and caveolin towards detergent extraction was demonstrated fully when different concentrations of TX-100 were tested for their effects on the integrity of PMs. At high detergent concentrations, a relatively small percentage of total G proteins was recovered in DRMs (about 20–30%). This portion rose to 70–80% when TX-100 concentration was lowered (Fig. 12, A and B; Table 1), but the decrease in detergent concentration had little effect on VSV-TRH-R-GFP recovery in DRMs (Fig. 13, Table 1). The relative content of VSV-TRH-R-GFP protein in DRMs did not exceed 5% of its total amount over the whole range of detergent concentrations (0.1–1%). Contrarily, a large portion of caveolin was detected in DRMs already at 1% TX-100, and this portion further increased to 90% in the presence of 0.25–0.1% TX-100 (Fig. 12C, Table 1). The same result was obtained for flotillin (Fig. 12D). These findings clearly support the conclusion that TRH receptor protein is excluded from DRMs.

When analyzing the TX-100 effect on PMs more closely, it was noted that the recovery of Gα subunits in DRMs was also dependent on the conditions of the extraction procedure, such as the way of mixing detergent with membrane suspension. With a final concentration of TX-100 of 1%, about 20–30% of Gα was recovered in DRMs when 1 ml of 2% TX-100 was added to 1 ml of cell homogenate and left to stand for 60 min at 0°C. On the other hand, addition of 1/10 volume (0.1 ml) of 10% TX-100 to cell homogenate (0.9 ml) resulted in 10–20% of Gα in DRMs. Thus, different recoveries of Gα subunits resulted even with the same final concentrations of TX-100 in the reaction mixture (data not shown).

VSV-TRH-R-GFP Receptor Protein Exhibits Similar Sensitivity to Detergent Extraction as Other Transmembrane Proteins—As already mentioned, the dominant portion of receptor protein was detected in the non-floating, high-density area of the gradient among detergent-solubilized and cytosolic components, and only a weak signal was detectable in DRMs (Figs. 8 and 13). This pattern of distribution was similar, if not identical, to that of numerous transmembrane PM markers (*23*). These markers include glycosylated (g) and non-glycosylated (ng) forms of CD147 (M6), MHCI (major histocompatibility complex, class I), CD29 (integrin β1 subunit), Tapa 1, the α subunit of Na,K-ATPase, the transmembrane form of CD58 (tCD58) and CD44 (lymphocyte homing receptor–phagocytic glycoprotein). Contrarily, GPI-bound proteins CD55 (complement decay accelerating factor), CD59 (complement protectin) and the GPI-bound form of CD58 (LFA-3) floated up and were recovered in DRMs (data not shown). The patterns of distribution of these marker proteins in HEK cells were both similar to those observed in human and mouse lymphocytes (*9*, *36*). In DRMs, a low signal of TRH-R and some transmembrane markers such as CD44 was clearly recognizable but difficult to quantify by immunoblot assays (*48*).

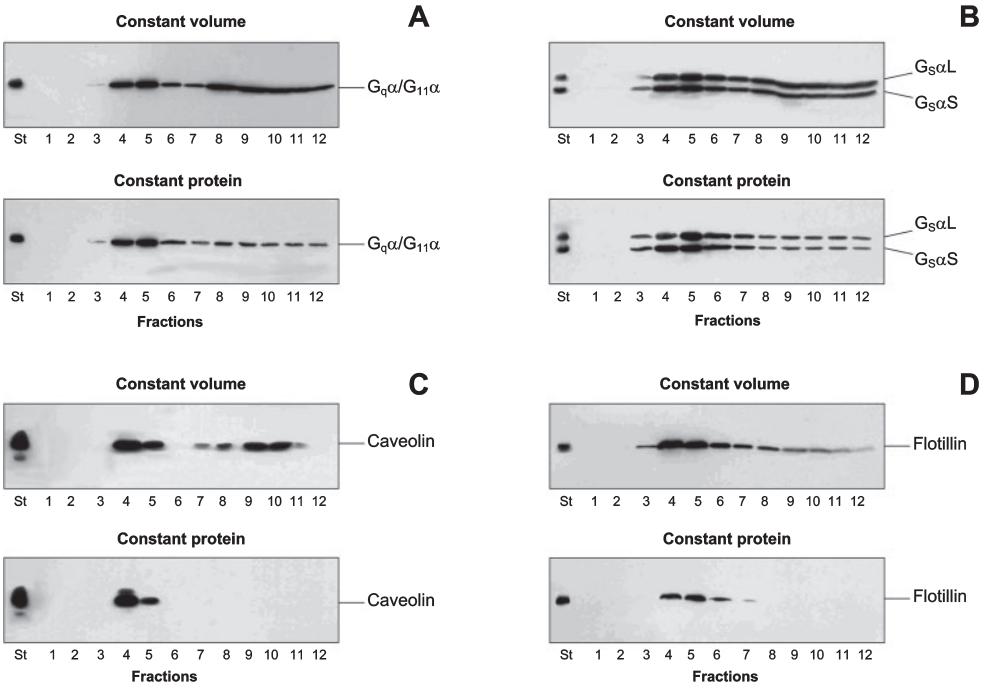

Fig. 9. **The "constant volume" *versus* "constant protein" mode of screening of the density gradients.** VSV-TRH-R-GFP expressing cells were cultivated in 6 flasks (80 cm^2 each), extracted in 1% Triton X-100 (60 min on ice) and fractionated by flotation in a sucrose density gradient (Beckman SW41 rotor) for 24 h at 39,000 rpm (187,000 × *g*). Gradient fractions (1 ml) were collected from the top to the bottom of the centrifuge tube and subjected to protein analysis. Distribution of $G_q\alpha/G_{11}\alpha$ (A), $G_s\alpha$ (B), caveolin (C) and flotillin (D) along the gradient was estimated in two ways: a *constant volume* of each fraction (75 μl, lower columns) or a *constant amount of protein* (15 μg, upper columns) was applied per gel, resolved by SDS-PAGE and analyzed by immunoblotting with antiserum CQ ($G_q\alpha/G_{11}\alpha$), Sigma G-5040 ($G_s\alpha$), C13630 (caveolin) and E35820 (flotillin-2/ESA) from Transduction Laboratories. St, rat brain microsomes or heart membranes, 20 μg.

TRH-R, Gα, Gβ and Caveolin Are Co-localized in Alkaline-treated/Sonicated Preparations of Membrane Domains. Detergent-Free Domains Resemble the Bulk of Plasma Membranes—The results presented in previous sections of this work reveal a big difference in the sensitivity of TRH-R-GFP, G proteins and caveolin towards detergent extraction. In original PMs, these molecules were localized together in 30–35% sucrose. When exposed to TX-100, caveolin was preferentially shifted up to DRMs (15–20% sucrose) (Figs. 5D and 11C); VSV-TRH-R-GFP protein was transferred down to the non-floating, detergent-solubilized area in 40% sucrose (Figs. 8 and 12). G proteins were distributed roughly 1:1 between the floating and non-floating regions of the gradient (Figs. 5, A–C, and 9, A–B; Table 1). This was not the case, however, with the gradient fractions obtained after alkaline treatment/sonication (*24*, *25*). This preparation of *membrane domains* resembled the bulk of PMs

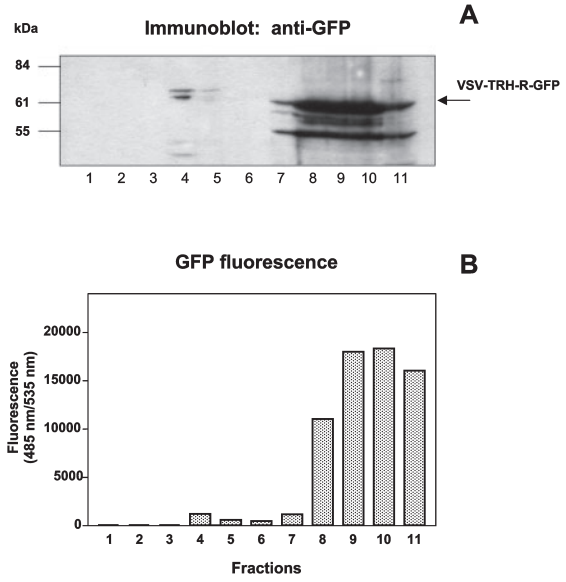

Fig. 10. **Double detection of VSV-TRH-R-GFP in density gradient fractions after TX-100 extraction.** HEK293 cells expressing VSV-TRH-R-GFP fusion protein were cultivated in 6 culture flasks (80 cm^2 each) and extracted with 1% TX-100. After fractionation of cell homogenate by flotation in sucrose density gradient, the receptor distribution was measured by immunoblotting using anti-GFP antibody (A) or by GFP fluorescence (B). These data represent a typical example selected from three experiments.

Table 1. **VSV-TRH-R-GFP, $G_q\alpha/G_{11}\alpha$ and caveolin solubility at different TX-100 concentrations.**

	TX-100	DRMs	Soluble
VSV-TRH-R-GFP	1.0%	<1%	>99%
	0.5%	2%	98%
	0.25%	3%	97%
	0.1%	9%	91%
$G_q\alpha/G_{11}\alpha$	1.0%	18%	82%
	0.5%	19%	81%
	0.25%	44%	56%
	0.1%	76%	24%
Caveolin	1%	50%	50%
	0.5%	61%	39%
	0.25%	87%	13%
	0.1%	90%	10%

VSV-TRH-R-GFP, $G_q\alpha/G_{11}\alpha$ and caveolin immunoblot signals detected in low-density fractions 2–6 (DRMs) or high-density fractions 8–12 (detergent soluble) were quantified by densitometric scanning and expressed as percentage of the total signal recovered in all fractions. Results represent the average of three experiments.

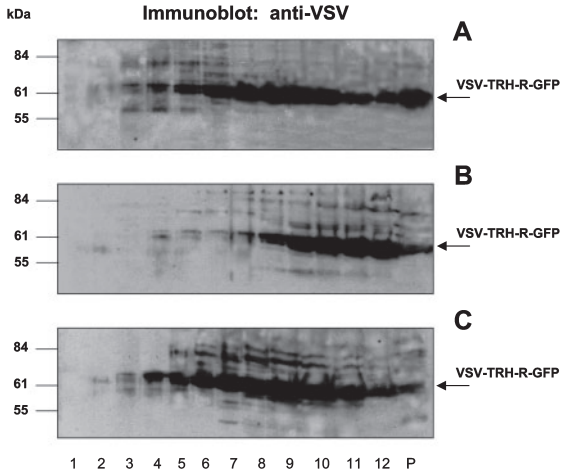

Fig. 11. **Distribution of VSV-TRH-R-GFP in density gradient fractions determined by immunoblotting with anti-VSV antibody.** Identical amounts of HEK293 cells (each corresponding to 6 × 80 cm^2) expressing VSV-TRH-R-GFP fusion protein were fractionated according to the homogenization (A), detergent extraction (B) or alkaline treatment/sonication (C) protocols. After fractionation of cell homogenates by flotation in sucrose density gradients, the distribution of VSV-TRH-R-GFP in all gradient fractions was assessed by immunoblotting with anti-VSV antibody. VSV-TRH-R-GFP was also determined in the gradient pellet (P), which was resuspedned in 1 ml of TME buffer. The immunoblots are representative examples of three similar experiments.

because VSV-TRH-R-GFP receptor protein (Figs. 4 and 8), Gα, Gβ subunit proteins as well as caveolin (Fig. 5, A–C, lower panels) were co-localized in fractions with only slightly lower buoyant density than the bulk of PM. The difference may be explained by the effect of sonication on the equilibrium position of the PM peak. Sonicated PM fragments were shifted to lower densities and distributed over a wider range of densities than observed in "homogenized only" preparations (Fig. 14).

The number of [^{3}H]TRH- and [^{3}H]ouabain-binding sites in alkaline-treated/sonicated PM fragments was higher than in PMs prepared by simple homogenization (Figs. 6 and 8). This was caused by the more efficient release of membrane material from the gradient pellet by vigorous disintegration of cell debris. In homogenized samples, the gradient pellet represented about 44% of homogenate protein; in alkaline-treated/sonicated preparations it was only 7% (Table 2). The increase of [^{3}H]ouabain binding in density gradient fractions was paralleled by its decrease in the gradient pellet; the total number of these binding sites was unchanged. On the other hand, the total number of [^{3}H]TRH binding sites rose significantly due to their better accessibility to hydrophilic ligands (approaching from aqueous phase), which was caused by treatment of membranes with highly alkaline solution of 0.5 M $NaHCO_3$ (pH 11).

DISCUSSION

Thyrotropin-Releasing Hormone Receptor and Other GPCRs—The absolute majority (>97%) of TRH receptor was excluded from detergent-resistant membrane domains/caveolae prepared by extraction with ice-cold TX-100.

Table 2. **Distribution of protein, [^{3}H]TRH and [^{3}H]ouabain binding sites in density gradient fractions (1–12) and in the pellet prepared from HEK293 cells expressing VSV-TRH-R-GFP fusion protein.**

	Homogenization	Alkaline treatment & sonication
Protein	mg (±SEM) (%)	mg (±SEM) (%)
Fractions 1–12	11.1 ± 1.4 (56)	19.4 ± 3.4 (93)
Pellet	8.7 ± 1.0 (44)	1.4 ± 0.5 (7)
Total	19.8 ± 2.1 (100)	20.8 ± 2.9 (100)
[^{3}H]TRH binding	pmol (±SEM) (%)	pmol (±SEM) (%)
Fractions 1–12	6.2 ± 2.2 (74)	14.1 ± 2.2 (99)
Pellet	2.2 ± 0.3 (26)	0.1 ± 0.1 (1)
Total	8.4 ± 2.3 (100)	14.2 ± 2.2 (100)
[^{3}H]ouabain binding	pmol (±SEM) (%)	pmol (±SEM) (%)
Fractions 1–12	16.0 ± 2.5 (70)	23.9 ± 1.1 (98)
Pellet	7.0 ± 3.1 (30)	0.5 ± 0.1 (2)
Total	24.0 ± 0.6 (100)	24.4 ± 1.2 (100)

Data represent the average of three independent fractionation procedures.

TRH receptor differed significantly from trimeric G proteins $G_q\alpha/G_{11}\alpha$ in its sensitivity towards this detergent. Receptor protein was almost totally solubilized and thus unable to float in density gradients, while $G_q\alpha/G_{11}\alpha$ and other Gα subunits were distributed almost equally between DRMs and the detergent-solubilized pool. In accordance with generally accepted view, caveolin and GPI-bound proteins were predominantly localized in DRMs/caveolae.

Our previous results on the localization of GPCRs in DRMs/caveolae indicated that no more than trace amounts of IP prostanoid receptors were present in these structures (*48*). The same result was obtained for other transmembrane proteins: glycosylated (g) and non-glycosylated (ng) forms of M6 (CD147), MHCI (major histocompatibility complex, class I), CD29 (integrin β1 subunit), Tapa 1, the α subunit of Na,K-ATPase, the transmembrane form of CD58 and CD44 (lymphocyte homing receptor–phagocytic glycoprotein). All these proteins behave in a similar way when exposed to TX-100. Therefore, we are rather sceptical about the presence of significant portion of the total cellular complement of GPCRs in DRMs/caveolae. This conclusion seems to be fully compatible with the dominant role of the non-caveolar, clathrin-mediated pathway in TRH-R signaling (*49–52*). It should be emphasized that TRH-R internalization is just one example of numerous GPCR signaling cascades where clathrin-mediated internalization represents the crucial pathway/mechanism of desensitization of hormone response (*53–55*). In contrast to GPCRs, hormone-induced internalization of $G_q\alpha/G_{11}\alpha$ proceeds rather slowly and is not affected by inhibitors of the clathrin-mediated pathway (*44*, *56*).

Nevertheless, numerous GPCRs have been identified in membrane domains/caveolae and described as moving in or out of these structures as a consequence of agonist stimulation (*44*, *56*). It was also shown that cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits and *vice versa* (*57*). From the perspective of our experimentation, these literature data suggest that

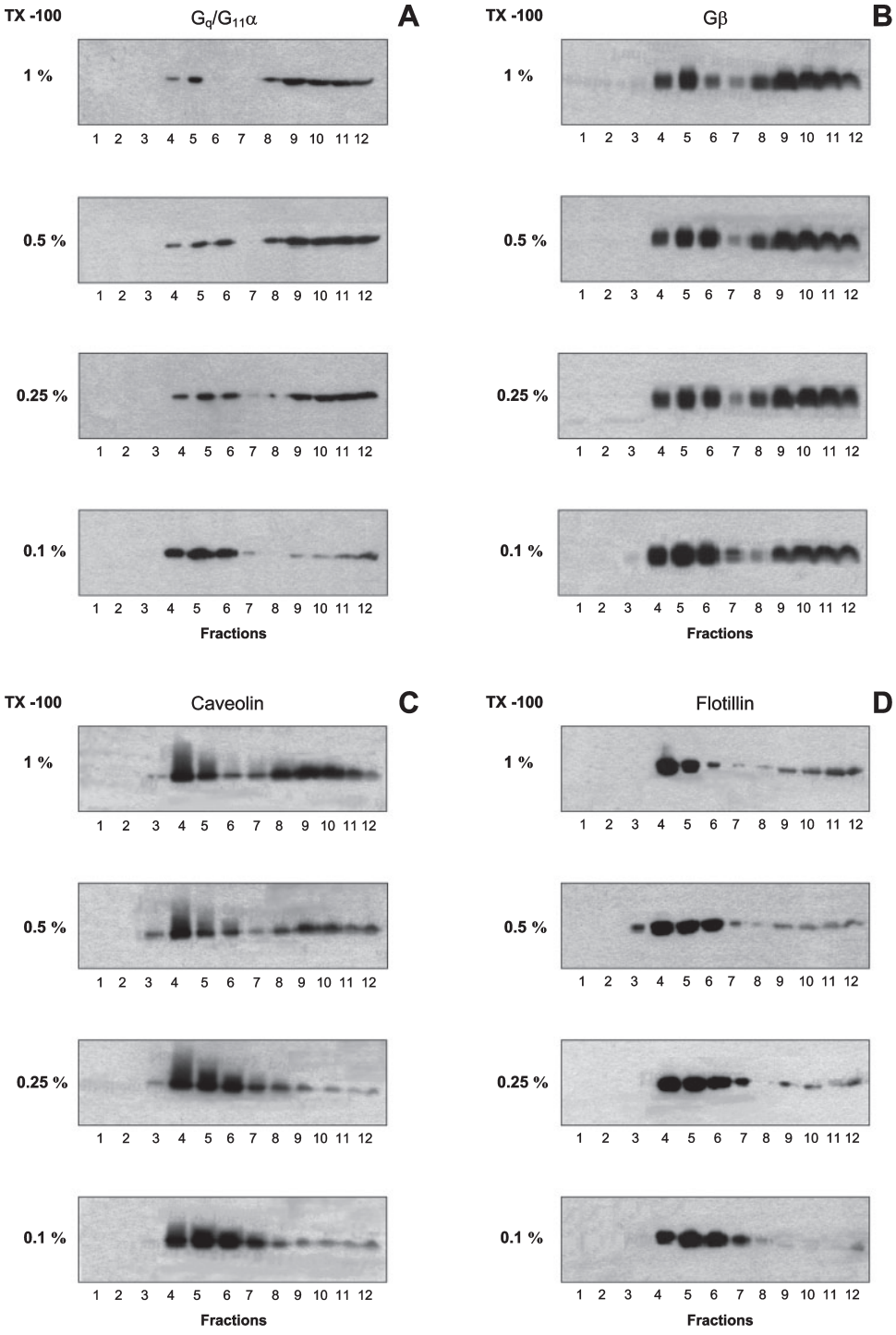

Fig. 12. **Effect of decreasing TX-100 concentrations on the density gradient distribution of Gq/G11α, Gβ, caveolin and flotillin.** HEK293 cells expressing VSV-TRH-R-GFP fusion protein were cultivated and in 24 flasks (80 cm^2 each), subdivided into four identical portions and fractionated according to the detergent extraction protocol using 1, 0.5, 0.25 or 0.1% TX-100. The individual protein molecules were identified by immunoblotting using constant (20 μl) aliquots of the gradient fractions.

the mere identification of GPCRs in DRMs/caveolae might be only of rather limited heuristic value unless it is complemented with information showing which part (expressed in percentage) represents the domain-bound pool of GPCRs among the total amount present in PMs. If a dominant portion of TRH-R is excluded from DRMs/caveolae it would be difficult to imagine how the caveolar pool of TRH-R could be the major determinant of TRH stimulation in naive cells.

Furthermore, when looking more closely into the real nature of lipid domains, a rather confusing and heterogeneous picture may arise. Caveolin and G proteins have been claimed to co-localize in DRMs. However, the use of more complex density gradients for the separation of detergent-resistant and detergent-soluble proteins reveals a clear difference in sensitivity to detergent extraction between these two types of signaling molecules (*23*). Moreover, the amount of $G_q\alpha/G_{11}\alpha$ in DRMs depends on TX-100 concentration (Fig. 12, Table 1) as well as on the way of screening the density gradients (Fig. 9). Therefore, more detailed methodological studies are needed to define lipid domains more clearly from the biochemical

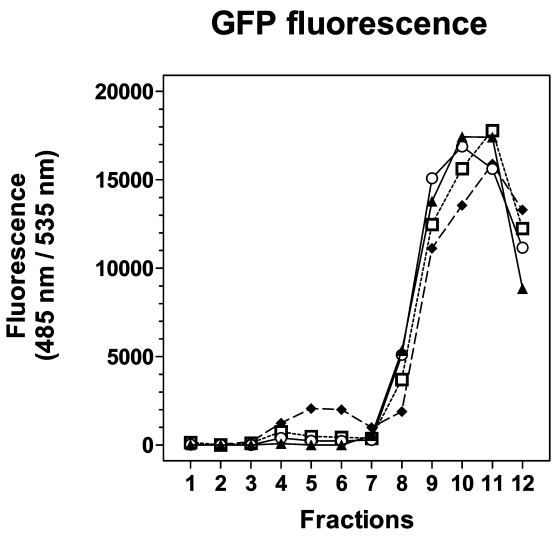

Fig. 13. **Effect of decreasing TX-100 concentrations on the density gradient distribution of VSV-TRH-R-GFP.** HEK293 cells expressing VSV-TRH-R-GFP fusion protein were cultivated in 24 flasks (80 cm^2 each), subdivided into four identical portions and fractionated according to the detergent extraction protocol using 1% (solid triangles), 0.5% (open circles), 0.25% (open squares) or 0.1% (solid diamonds) TX-100. GFP fluorescence (E_x 485 nm/E_m 535 nm) was measured in constant (100 μl) aliquots of the gradient fractions. Results shown are representative of a single fractionation procedure.

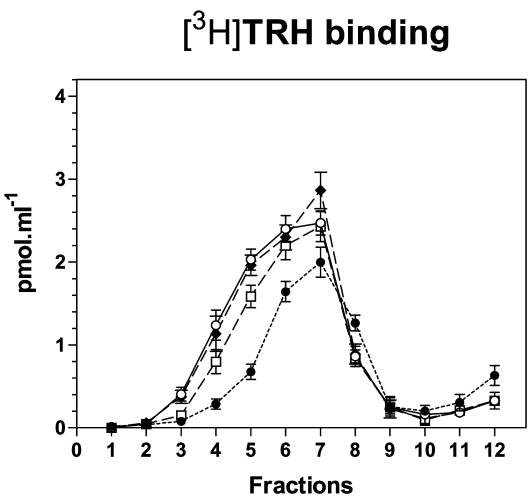

Fig. 14. **Effect of sonication on equilibrium position of PM fragments in the density gradients.** HEK293 cells were cultivated in 24 flasks (80 cm^2 each), homogenized and subdivided into four identical portions. (Solid circles), homogenization only; homogenization followed by sonication using increasing output intensity (open squares, solid diamonds, open circles) in Bandelin sonoplus sonicator (Germany). Two ml of cell homogenate was placed in a 15-ml conical tube (in a mixture of crushed ice and water, 0°C) and exposed to five subsequent 10-s pulses with force set at 10% (open squares), 20% (solid diamonds) and 40% (open circles) of maximum output; each pulse was followed by a 60-s break to ensure perfect cooling. The distribution of PM was assessed by a [^{3}H]TRH binding study (see "MATERIALS AND METHODS"). Results shown are representative of a single fractionation procedure measured in triplicate.

point of view. The current definition of DRMs is strictly methodological, and the lipid shell hypothesis may be applied to a rather limited number of inherently low-density proteins surrounded by cholesterol and glycolipids (*58*). A comprehensive understanding of GPCR function in such elusive structures as lipid domains (for recent reviews see Refs. *59* and *60*) would require application of the same methods for their isolation.

The question of co-localization of a given protein molecule with caveolin may present a controversial issue, especially in detergent-free preparations of membrane domains. These structures do not seem to be anything other than small, low-density fragments of PMs artificially formed by sonication and/or drastic homogenization. Caveolin, GPCRs and G proteins are all present in these fragments together. This statement is valid for interpretation of the results of our previous study dealing with δ-opioid receptors (*26*), and it may be applicable to density gradient profiles obtained from primary cultures of cardiac myocytes (*61*, *62*). This approach is obviously also relevant for interpreting our present data. Application of alkaline treatment (0.5 M $NaHCO_3$, pH 11) does not substantially alter this picture; one of the drawbacks of this technique is that the highly alkaline environment (pH 11) radically diminishes adenylyl cyclase activity. In alkaline-treated and sonicated *domains*, TRH-R, $G_q\alpha/G_{11}\alpha$ and caveolin are co-localized. If sonicated mildly, these fragments retain the functional coupling between GPCRs and G proteins, which can be measured as agonist-stimulated GTPγS binding (*26*). From this point of view, the more simple procedure for preparation of membrane domains introduced by Smart *et al.* (*63*) seems to be better suited for future studies focused on the structure–function correlation in *domain / raft* compartments. It remains to be clarified in the future whether the fashionable, "up-to-date" picture of domain heterogeneity reflects something more than the methodological variability in preparation of different types of PM fragments.

Long-Term Agonist Stimulation and G Proteins in DRMs—In the TRH-R and $G_q\alpha/G_{11}\alpha$-mediated signaling cascade, hormone-induced internalization of $G_q\alpha/G_{11}\alpha$ proteins is much slower than internalization of the receptor molecules. Whereas TRH-R is internalized within minutes (*49–52*, *64*), internalization of $G_q\alpha/G_{11}\alpha$ is manifested fully after several hours of TRH stimulation (*44*, *65*, *66*). Internalization of $G_q\alpha/G_{11}\alpha$ is not affected by inhibitors of the clathrin-dependent pathway. Data presented in our present work indicate that TRH-R and $G_q\alpha/G_{11}\alpha$ proteins are localized in different PM compartments. These compartments are distinguished by different sensitivity towards the non-ionic detergent TX-100. Whereas TRH-R is excluded from DRMs/caveolae, a significant portion of $G_q\alpha/G_{11}\alpha$ is present in these structures.

Our previous studies indicated that prolonged stimulation of TRH and IP prostanoid receptors results in specific depletion of the membrane domain-bound pool of the cognate G proteins $G_q\alpha/G_{11}\alpha$ and $G_s\alpha$, respectively (*23*, *48*). The level of other G proteins was unchanged. The 2-D electrophoretic resolution and immunoblot detection of PM markers indicated that this depletion proceeded without major (non-specific) alteration of protein composition of DRMs/caveolae (*67*). In general, the functional significance of association of trimeric G proteins with DRMs/caveolae/raft/domain compartment(s) is unclear.

One theory holds that DRMs/caveolae serve as compartment(s) for recruitment of the components of different signaling pathways to increase efficiency and/or speed of the functional coupling between receptor(s) and effector(s); an alternative theory is that caveolae attract the components of signaling cascades to desensitize or terminate the receptor signal (*1–4*, *6–8*, *68*). The latter possibility has gained substantial support, as the stimulation of $G_q\alpha/G_{11}\alpha$ by cholecystokine or substance P, of $G_i3\alpha$ by cyclopentyladenosine or of both $G_q\alpha/G_{11}\alpha$ and $G_i3\alpha$ by acetylcholine, specifically increased the content of the respective G proteins in caveolin immunoprecipitates (*20*). Sequestration of G protein subunits by binding to caveolin correlated with heterologous desensitization to a given agonist. Furthermore, Oh and Schnitzer (*69*) described a specific segregation of $G_q\alpha/G_{11}\alpha$ proteins into caveolae even under control conditions, which may imply that activation of the G proteins has to proceed either as competition between activated receptors and caveolin or as removal of $G_q\alpha/G_{11}\alpha$ from caveolae.

Superficially, the results of Murthy and Makhlouf (*20*) may seem to contradict our present data. Nevertheless, the possibility of an overlap may arise from a closer view. According to Murthy and Makhlouf (*20*), the increase of $G_q\alpha/G_{11}\alpha$ and $G_i3\alpha$ in caveolin immunoprecipitates is transient; the maximum is reached between 10 and 20 min of agonist exposure and further stimulation results in decrease of G protein levels. After 60 min, caveolar G proteins return to control levels. We were not able to demonstrate any significant change of $G_q\alpha/G_{11}\alpha$ or $G_s\alpha$ in DRMs isolated from TRH-R, VSV-TRH-R-GFP or FhIPR expressing cells after 5–30 min of agonist stimulation (data not shown). This might be due to the fact that DRMs represent a much broader spectrum of membrane fragments than those gathered in caveolin immunoprecipitates. However, some redistribution of G proteins after short-term stimulation was manifested by the clustering and appearance of patches of fluorescent material corresponding to doubly immunostained $G_q\alpha/G_{11}\alpha$ on the surface of HEK293 cells (*44*). In S49 lymphoma and HEK293 cells, we noticed a transition of about 5–10% of plasma membrane $G_s\alpha$ and $G_q\alpha/G_{11}\alpha$ to low-density membrane fragments (*27*, *41*, *43*). Therefore, the short-term (transient) shift of trimeric G proteins to caveolin immunoprecipitates (*18*, *20*) or permanent localization of $G_q\alpha/G_{11}\alpha$ in caveolae (*69*) may represent preceding step(s) in the reaction sequence, which at longer times of agonist stimulation results in depletion of an overall pool of G proteins in DRMs. The decrease of G proteins in DRMs does not seem to arise from some artificial situation because (i) it was demonstrated in two different signaling cascades, (ii) it proceeds as a receptor-specific process (*23*, *48*), (iii) it was demonstrated in DRMs as well as in detergent-free (alkaline-treated) preparations of membrane domains, and (iv) it proceeds without any major protein reorganization of domain structure (*67*).

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