Thyrotropin-releasing hormone-induced depletion of $G_q\alpha/G_{11}\alpha$ proteins from detergent-insensitive membrane domains

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Abstract The role of detergent-insensitive membrane domains (DIMs) in desensitisation of the G protein-coupled receptor-mediated hormone response was studied in clone E2M11 of HEK293 cells which stably express high levels of both thyrotropin-releasing hormone (TRH) receptors and $G_{11}\alpha$ G protein. DIMs were prepared by flotation in equilibrium sucrose density gradients and characterised by a panel of membrane markers representing peripheral, glycosylphosphatidylinositol-bound as well as integral membrane proteins (caveolin, CD29, CD55, CD59, CD147, the α subunit of Na,K-ATPase) and enzyme activities (alkaline phosphatase, adenylyl cyclase). Caveolin-containing DIMs represented only a small fraction of the overall pool of $G_q\alpha/G_{11}\alpha$-rich domains. Prolonged stimulation of E2M11 cells with TRH resulted in dramatic depletion of $G_q\alpha/G_{11}\alpha$ from all DIMs, which was paralleled by a concomitant $G_q\alpha/G_{11}\alpha$ increase in the high-density gradient fractions containing the bulk-phase membrane constituents soluble in 1% Triton X-100. Distribution of membrane markers was unchanged under these conditions. Membrane domains thus represent a substantial structural determinant of the G protein pool relevant to desensitisation of hormone action.

Key words: Desensitization; Thyrotropin-releasing hormone; Detergent-insensitive membrane domain; $G_q\alpha/G_{11}\alpha$ protein; Caveolae

1. Introduction

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) may be divided into 'membrane-bound' and 'cytosolic' forms, the former type meaning those which may be recovered from particulate, membrane fractions and the latter type meaning those G protein α subunits which may be recovered from the soluble, cytosol fraction (250 000 × *g* supernatant) [1]. Plasma membrane G proteins represent the dominant portion of the particulate, membrane-bound pool, but heterotrimeric G proteins also have been detected in 'non-plasma membrane' compartments such as light-vesicles (endosomes), Golgi apparatus and endoplasmic reticulum vesicles [2–6]. Sustained hormone stimulation of target cells results in desensitisation of hormone response which can be associated with internalisation, subcellular intermembrane transfer and solubilisation of trimeric G proteins [1,3,4,6–10].

The plasma membrane pool of G proteins may be further divided according to detergent solubility. The bulk of G proteins are solubilised in detergents such as Triton X-100 (TX-100) (1%, 0°C, 30–60 min) along with bulk-phase membrane lipids and most other plasma membrane proteins. However, a significant fraction of G proteins has also been found in detergent-insoluble membrane domains (DIMs) ([11–13], for review, see [1]). As the physiological significance of domain-bound G proteins remains unknown, we decided to analyse hormone-induced changes between 'domain-bound' and 'bulk-phase' membrane forms of the G proteins G_q/G_{11} with special attention to caveoli [15], which have previously been suggested to be significant determinants of hormone action.

2. Materials and methods

2.1. Chemicals

Tissue culture reagents and media were supplied by Gibco BRL (Renfrewshire, UK) or Sevac (Prague, Czech Republic). All other chemicals and drugs, including geneticin and hygromycin, were purchased from Sigma (St. Louis, MO, USA). Caveolin-oriented antisera C13630 and C37120 were purchased from Transduction Laboratories (Nottingham, UK).

2.2. Cell culture

E2M11-human embryonic kidney (HEK) 293 cells (clone expressing high amounts of both thyrotropin-releasing hormone (TRH) receptor and $G_{11}\alpha$ protein) were cultivated in Dulbecco's modified Eagle's medium supplemented with 8% (v/v) heat-inactivated new-born calf serum, geneticin (800 μg/ml) and hygromycin B (200 μg/ml) at 37°C under 5% CO_2 atmosphere as described previously [4].

2.3. Isolation of detergent-resistant membrane domains (DRMs)

E2M11 cells were harvested from 6×75 cm^2 flasks and collected by low-speed centrifugation at 1800 rpm for 10 min. The resulting sediment was resuspended by repipetting in 2 ml 1% TX-100, 3 mM $MgCl_2$, 1 mM EDTA, 50 mM Tris–HCl, pH 7.5, and 1 mM phenylmethylsulfonyl fluoride (TX buffer). After 45 min at 0°C, 2 ml of 1% TX-100 extract was mixed with 2 ml of ice-cold 80% w/v sucrose, transferred into a Beckman SW 41 rotor centrifuge tube and overlaid with 35, 30, 25, 20, 15, 10 (1 ml each) and 5% w/v sucrose (1.5 ml). Sucrose density gradient fractions 1 (1 ml, 5%), 2 (1 ml, 5/10%), 3 (1 ml, 10/15%), 4 (1 ml, 15/20%), 5 (1 ml, 20/25%), 6 (1 ml, 25/30%), 7 (1 ml, 30/35%), 8 (1 ml, 35/40% sucrose interphase) and 9–11

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Abbreviations: DIM, detergent-insensitive membrane domain; G proteins, heterotrimeric guanine nucleotide-binding regulatory proteins; $G_q\alpha/G_{11}\alpha$, α subunits of the G proteins G_q/G_{11}; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; GPI, glycosylphosphatidylinositol; Na,K-ATPase, sodium plus potassium-activated, ouabain-dependent adenosine triphosphatase (EC 3.6.1.3); TRH, thyrotropin-releasing hormone; TBS, Tris-buffered saline

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(1 ml, 40% sucrose) were collected manually from the meniscus after centrifugation for 24–28 h at 36 000 rpm (96 508 × *g*).

2.4. Production of antisera

$G_q\alpha/G_{11}\alpha$ oriented antiserum 452 is rabbit polyclonal antiserum which was raised in rabbits obtained from Velaz (Prague, Czech Republic). Rabbits were immunised with a synthetic peptide QLNLKEYLNV (C-terminal decapeptide of $G_q\alpha/G_{11}\alpha$) conjugated to key-hole limpet hemocyanin (Calbiochem) as described previously [8]. Membrane protein markers CD29, CD55, CD59 and CD147 were detected with a panel of monoclonal antibodies obtained from the collection of Prof. Dr. V. Hořejší (Institute of Molecular Genetics AV CR, Prague, Czech Republic). Sodium plus potassium-activated, ouabain-dependent adenosine triphosphatase (Na,K-ATPase) was detected by affinity-purified polyclonal antiserum which was a kind gift of Dr. R. Antolowich, Giesen University, Germany.

2.5. Immunoblotting 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) and solubilised in Laemmli buffer. Standard (10% w/v acrylamide/0.26% w/v bis-acrylamide) or high-resolution urea (12.5% w/v acrylamide/0.0625% w/v bis-acrylamide containing 6 M urea) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described before in detail [3]. 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 in 4% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 8.0, 150 mM NaCl). The 452 antiserum was added in 1% (w/v) BSA in TBS containing Tween 20 (0.2% v/v) and incubated for at least 2 h. The primary antibody was then removed and the blot washed extensively in TBS with 0.2% (v/v) Tween 20. Secondary antiserum (goat anti-rabbit IgG conjugated with alkaline phosphatase) was applied for 1 h and after three 10 min washes, the blots were developed in TNM buffer (100 mM Tris–HCl, pH 9.0, 100 mM NaCl and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate (100 μg/ml) and nitroblue tetrazolium (200 μg/ml) as substrate [6]. The developed blots were scanned with an imaging densitometer Astra 610P (UMAX) to enable quantification of the immunoblots.

2.6. Detection of selected plasma membrane receptor complexes

Glycosylphosphatidylinositol (GPI)-anchored proteins CD55 (complement decay accelerating factor) and CD59 (complement protectin) as well as GPI-non-anchored proteins CD29 (integrin β_1 subunit) and CD147 (M6) were detected by immunoblotting with specific monoclonal antibodies and the immunoblot signals were detected by ECL technique according to well established protocols [16]. The classical marker of integral membrane proteins, the α subunit of Na,K-ATPase, was detected by antibodies purified by protein A-affinity chromatography from rabbit polyclonal antiserum, which was prepared against the isolated α_1 subunit of this. The immunoblot signal was developed with indolyl phosphate and nitroblue tetrazolium as described above [6].

2.7. Determination of membrane marker enzyme activities

Adenylyl cyclase was determined by the method of Salomon et al. [17], which involves the separation of cyclic [^{32}P]AMP from [α-^{32}P]ATP on sequential columns of Dowex 50 and alumina. Briefly, density gradient fractions (constant volume of 50 μl) were incubated for 30 min at 37°C in a total volume of 0.1 ml of 70 mM HEPES/HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EDTA, 16 μg/ml pyruvate kinase, 10 mM potassium phosphoenolpyruvate, 160 μg/ml BSA, 0.1 mM ascorbic acid, 0.01 mM RO-201724 (phosphodiesterase inhibitor) and 0.15 mM ATP plus [α-^{32}P]ATP (about 1×10^6 cpm per sample). The activating ligands forskolin (50 μM) or $MnCl_2$ (10 mM) were added as described. The 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 (about 20 000 cpm per assay as internal standard) and heating for 5 min at 95°C.

Alkaline phosphatase (EC 3.1.3.1) was determined according to DeChatelet and Cooper [18] using *p*-nitrophenylphosphate as substrate.

3. Results

3.1. Caveolin-containing DIMs are not identical with $G_q\alpha/G_{11}\alpha$-containing DIMs

Caveolin (about 40% of total cellular pool) was detected in fractions 3 and 4 which represented interphases between 10/15% and 15/20% w/v sucrose solutions, respectively (Fig. 1A). The dominant localisation of caveolin in these low-density fractions could also be documented by 'specific content' ratio (Fig. 1B), relating the intensity of the immunoblot signal to the amount of protein present in a given fraction. This ratio in fractions 3 and 4 was three times higher than in fraction 5 and at least 10 times higher than in any other gradient fraction. This type of localisation of *caveolae* (caveolin-containing domains) on equilibrium sucrose density gradients corresponded well to the previously published data observed in other cell types [13,19].

Proteins reacting with caveolin antiserum and exhibiting the same relative mobility on SDS-polyacrylamide gels as caveolin were also detected in the high-density, non-floating region of the gradient (Fig. 1A). The high-density pool of caveolin represented 50% of the total amount recovered from the gradient.

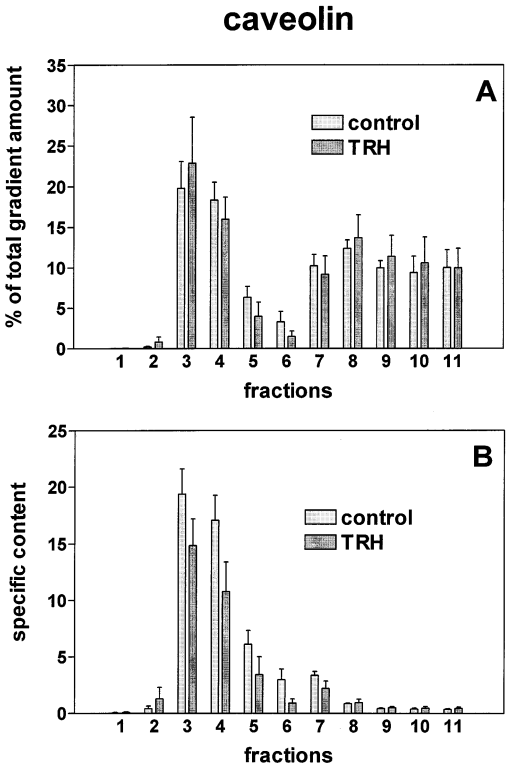

Fig. 1. Caveolin distribution in density gradient fractions. E2M11-HEK293 cell monolayers (roughly 80% confluent) were incubated with (1 μM, 2 h) or without TRH, scraped into ice-cold 1% v/v TX-100 in Tris buffer, pH 7.5, and caveolar and DIG membranes isolated by density gradient centrifugation. Gradient fractions were TCA-precipitated and analysed by immunoblotting as described in Section 2. (A) Caveolin content in gradient fractions was expressed as percentage of the total immunoblot signal detected in fractions 1–11. (B) Immunoblot signal of caveolin in a given fraction (expressed as percentage of total signal in fractions 1–11) was divided by the percentage of protein detected in this fraction. The data represent an average ± S.E.M. obtained from five independent fractionation procedures. The difference between control and hormone-treated samples analysed by a Student's *t*-test was not significant for any of the gradient fractions ($P > 0.05$).

The 'specific content' of caveolin in these fractions (8–11), however, was much lower than in the low-density area of the gradient (Fig. 2B). This was a reflection of the fact that fractions 8–11 contained as much as 3–6 mg protein per ml, while the protein content in low-density fractions 2–6 was extremely low (0.04–0.09 mg per ml) (data not shown).

The density gradient profiles of $G_q\alpha/G_{11}\alpha$ proteins were substantially different from those obtained for caveolin (Fig. 2). With the exception of fraction 1 (5% sucrose), $G_q\alpha/G_{11}\alpha$ proteins were detected in all gradient fractions and in multiple DIMs ranging in density from 10 to 35% sucrose. The majority of $G_q\alpha/G_{11}\alpha$ was detected in the high-density area of the gradient representing 40% sucrose (fractions 8–11). By comparing the $G_q\alpha/G_{11}\alpha$ content in the 'floating', low-density region (DIMs, fractions 1–6) with the 'non-floating', high-density region (bulk-membrane phase, fractions 8–11) of the gradient, it was estimated that 60–70% of the total cellular pool of these G proteins is localised in the high-density region. This localisation indicates that the majority of $G_q\alpha/G_{11}\alpha$ is detergent(1% TX-100)-soluble. The 'TX-100-soluble' character of $G_q\alpha/G_{11}\alpha$ detected in high-density region was also evidenced by high-speed centrifugation of fractions 8–11 which were pooled and diluted with water 1:1 (Fig. 3). Less than 10% of $G_q\alpha/G_{11}\alpha$ proteins was transferred to the sediment after this centrifugation.

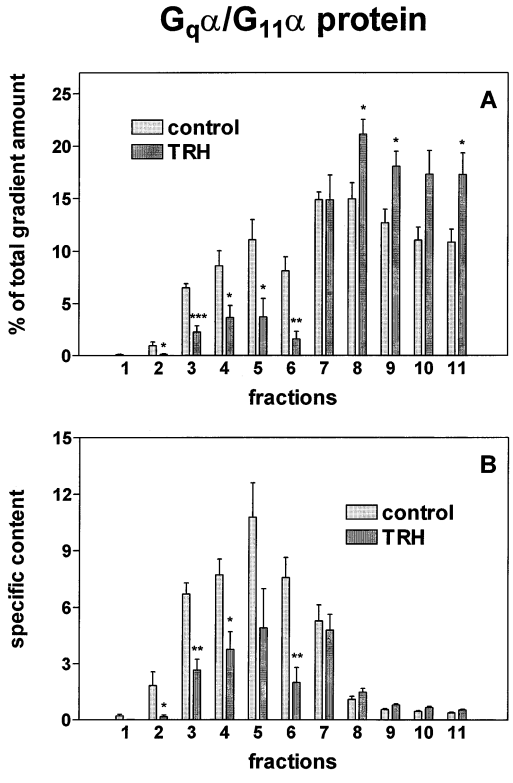

Fig. 2. TRH-induced change in distribution of $G_q\alpha/G_{11}\alpha$ proteins. E2M11-HEK cells were exposed to TRH (1 μM, 2 h), treated with 1% TX-100 and fractionated as described in the legend to Fig. 1. The $G_q\alpha/G_{11}\alpha$ content in gradient fractions was measured in parallel samples of TCA precipitates used for caveolin detection. (A) $G_q\alpha/G_{11}\alpha$ content in gradient fractions expressed as percentage of the total immunoblot signal detected in fractions 1–11. (B) Immunoblot signal of $G_q\alpha/G_{11}\alpha$ (expressed as percentage of total signal in fractions 1–11) was divided by the percentage of protein detected in this fraction. The data represent an average ± S.E.M. obtained from five independent fractionation procedures. The difference between control and hormone-treated samples was analysed by a Student's *t*-test; ***, **, * indicates significant effect of cold acclimation ($P < 0.001$, $P < 0.01$, $P < 0.05$, respectively).

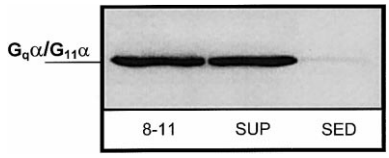

Fig. 3. $G_q\alpha/G_{11}\alpha$ in high-density region of the gradient represents TX-100-soluble proteins. High-density fractions 8–11 were diluted with water 1:1 and centrifuged at 75 000 rpm (250 000 × *g*) for 45 min in a Sorvall RC-M100 centrifuge. The same volumes of resulting supernatant and of the original (diluted) fractions were TCA-precipitated and analysed for $G_q\alpha/G_{11}\alpha$ content by immunoblotting. The data represent a typical experiment. 8–11, gradient fractions 8–11; SUP, 250 000 × *g* supernatant; SED, 250 000 × *g* sediment.

3.2. *DIMs-bound $G_q\alpha/G_{11}\alpha$ is depleted by prolonged TRH treatment*

Prolonged stimulation of E2M11-HEK cells with TRH

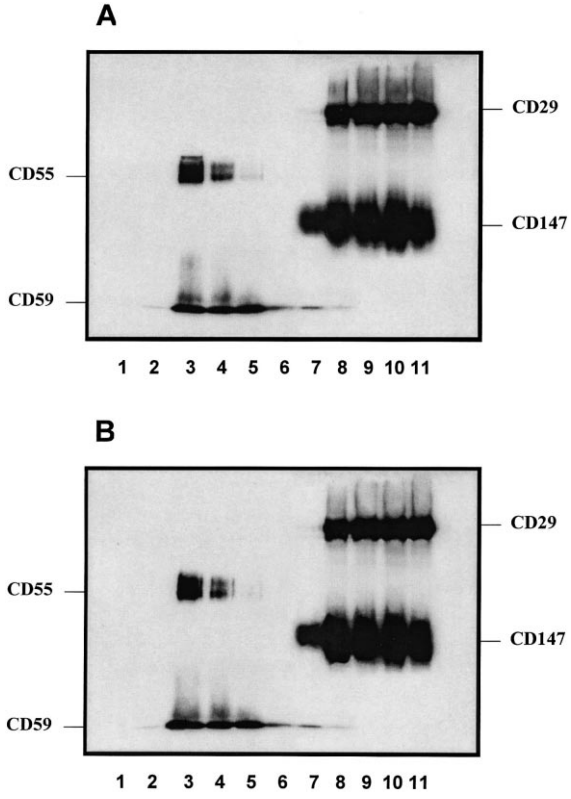

Fig. 4. CD receptor complexes as markers of GPI-bound and integral membrane proteins. CD29, CD55, CD59 and CD147 receptor complexes were determined in density gradient fractions 1–11 prepared from control (A) or TRH (1 μM, 2 h)-exposed (B) E2M11 cells by immunoblotting with the specific monoclonal antibodies (see Section 2). The data represent a typical experiment.

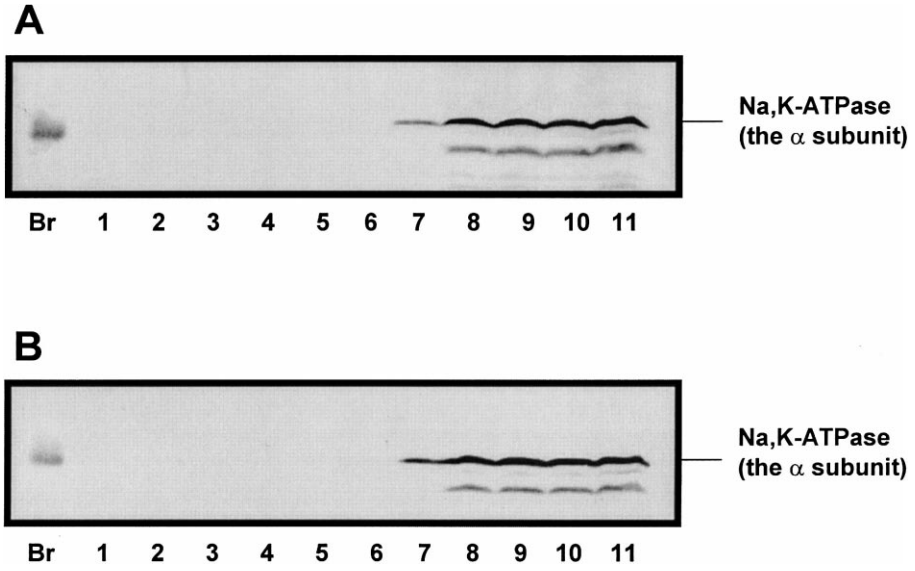

Fig. 5. Na,K-ATPase (sodium+potassium-activated, ouabain-sensitive adenosinetriphosphatase). Density gradient profile of Na,K-ATPase was detected by immunoblotting with specific antibodies (protein A-purified), which were raised in rabbits against the isolated α_1 subunit of this enzyme. Fractions 1–11 were prepared from control (A) or TRH (1 μM, 2 h)-treated (B) cells as described before. The results show a typical immunoblot from one representative fractionation procedure. Br: brain microsomes, 20 μg protein.

(1 μM, 2 h) induced a dramatic redistribution of $G_q\alpha/G_{11}\alpha$ proteins within the density gradient fractions (Fig. 2). The $G_q\alpha/G_{11}\alpha$ content in low-density fractions 1–6 was decreased more than 50%, while the amount of these G proteins in high-density fractions 8–11 was significantly increased (by about 30%). Thus, long-term agonist stimulation induced a transfer of G proteins from DIMs to the detergent-sensitive membrane compartments representing the bulk-phase membrane constituents.

3.3. *Distribution of membrane markers is unchanged by agonist stimulation*

Distribution of caveolin (Fig. 1), CD29, CD55, CD59, CD147 (Fig. 4) and Na,K-ATPase (Fig. 5) was not affected by TRH treatment (compare A and B). This finding indicates that prolonged stimulation of E2M11-HEK293 cells by TRH does not alter the overall distribution of membrane markers and thus, hormone-induced change in distribution of $G_q\alpha/G_{11}\alpha$ is specifically related to hormonal challenge and desensitisation.

3.4. *Domain-bound (low-density) versus detergent-soluble (high-density) membrane proteins*

Surprisingly, the gradient distribution of the CD receptor markers in E2M11-HEK293 cells (Fig. 4) was similar, if not identical, to that found in T-lymphocytes [20]. GPI-bound CD55 was located exclusively in caveolae (fraction 3). CD59 was detected in a wide range of DIMs ranging in density from 10 to 35% sucrose and thus exhibited similar distribution to that described above for $G_q\alpha/G_{11}\alpha$. Integral, transmembrane proteins CD29 and CD147 were detected exclusively in the high-density area of the gradient. Localisation of CD29 and CD147 in high-density area of the gradient indicated a 'detergent-soluble' state of these proteins, which was described before for other CD markers (Černý and Brdička, unpublished; [21]). It may therefore be concluded that GPI-anchored proteins from HEK293 cells, in accordance with earlier results on T-cells [20,21], were concentrated in DIMs, but integral, transmembrane proteins were preferentially localised in 'non-floating', high-density region of the gradient.

Similar results were obtained from an immunoblot analysis

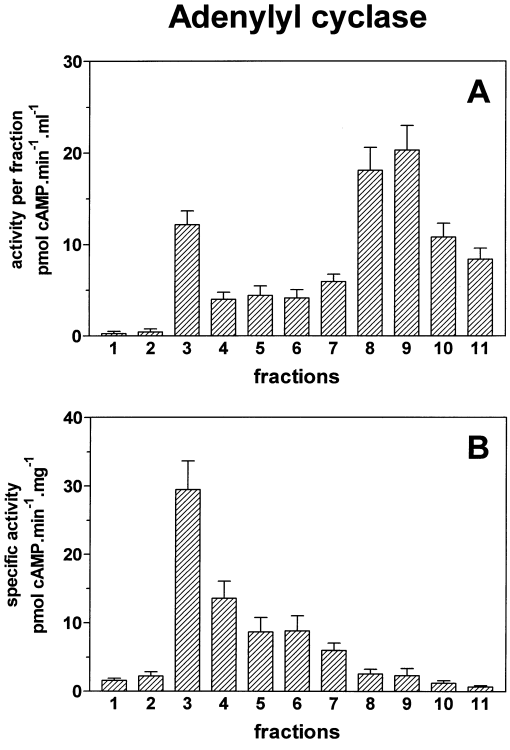

Fig. 6. Adenylyl cyclase. Constant volume of 50 μl of gradient fractions 1–11 was used as a source of an enzyme and manganese (10 mM)-stimulated adenylyl cyclase activity was determined as described in Section 2. A, total activity per fraction = pmol cAMP/min/ml; B, specific enzyme activity = pmol cAMP/min/mg. The results shown represent an average ± S.E.M. from three independent isolation procedures.

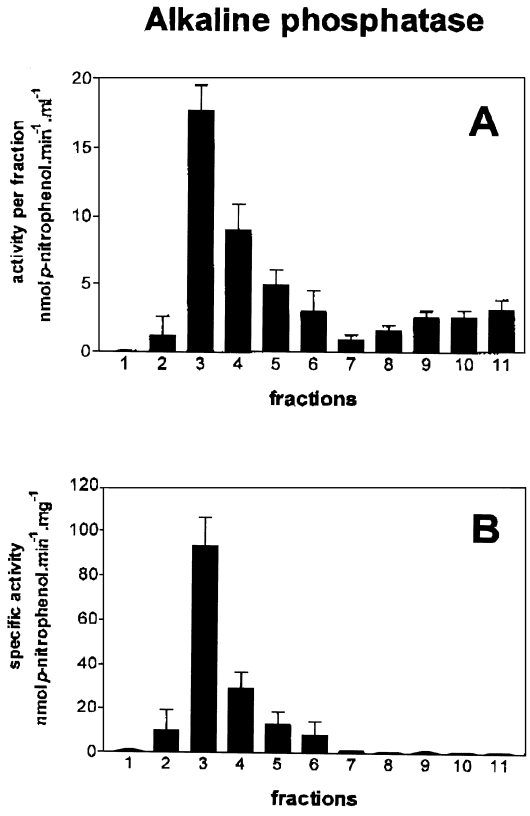

Fig. 7. Alkaline phosphatase. Constant volume of 10 μl of gradient fractions 1–11 was used as source of enzyme and alkaline phosphatase activity was measured as *p*-nitrophenol production using *p*-nitrophenylphosphate as substrate (see Section 2). A, total activity per fraction = nmol *p*-nitrophenol/min/ml; B, specific enzyme activity = nmol *p*-nitrophenol/min/mg. The results shown represent an average ± S.E.M. from three independent isolation procedures.

of Na,K-ATPase α subunit and measurement of adenylyl cyclase enzyme activity (Figs. 5 and 6). The α subunit of Na,K-ATPase, which represents a highly hydrophobic, integral membrane protein, was located exclusively in the high-density area of the gradient (Fig. 5). Manganese-stimulated adenylyl cyclase exhibited a dual localisation. A major part of adenylyl cyclase (based on cAMP production per fraction data: cAMP/min/ml; Fig. 6A) was localised in the high-density area in fractions 8 and 9. A minor portion of this enzyme activity was detected in the 'domain-rich', low-density region of the gradient, in fractions 3 and 4. The DIMs-bound pool of adenylyl cyclase became dominant when cAMP production was expressed per mg protein present in a given fraction: cAMP/min/mg protein (Fig. 6B). The specific activity of Mn^{2+}-stimulated adenylyl cyclase in caveolin-rich fraction 3 was 15 times higher than in the 'soluble', high-density fractions 8 and 9. Almost identical distribution was found for forskolin-stimulated adenylyl cyclase (data not shown).

In contrast to adenylyl cyclase, there was almost no alkaline phosphatase activity in the high-density region of the gradient. This enzyme activity was located preferentially in caveolin-containing fraction 3 (Fig. 7). From this point of view, alkaline phosphatase thus exhibited the same characteristics as caveolin and GPI-bound CD55 receptor. These findings are again in good agreement with previous literature data [14,15], indicating a close association of alkaline phosphatase with caveolae and/or DIMs.

4. Discussion

Extensive stimulation of cells caused by persistent activation of the signal transduction pathways initiated by G protein-coupled receptors (GPCRs) can lead to pathological effects. Persistent activation of adenosine type-2 receptor in thyroid gland leads to cell hyperplasia and hyperthyroidism [22]; activating mutations of G proteins may cause pituitary tumours [23,24]. In defence against excessive stimulation, cells have evolved a number of autoregulatory mechanisms which are initiated rapidly after GPCR activation and serve to dampen the stimulated response. Desensitisation, which is the term applied to this overall process, may be defined as decreased cellular response to a stimulus of constant strength [25].

Desensitisation of GPCRs may be produced by both rapid and slow mechanisms. The former take place within seconds to minutes after receptor activation, while the latter do not become manisfestant until minutes to hours after activation. Among the long-term mechanisms of desensitisation of hormone action, agonist stimulation of target cells was found to result in *solubilisation* of G protein α subunits, i.e. in transfer from plasma membranes to cytosol fraction (supernatant 250 000 × *g*) [1]. It has also been shown that prolonged stimulation of target cells results in transfer of G proteins from plasma membranes to light-vesicular (endosomes) membrane compartments (*subcellular intermembrane redistribution*) [3–6,26]. More recently, isoproterenol- or thyrotropin-releasing hormone (TRH) stimulation of transfected cell lines has been found to be associated with *internalisation* of $G_s\alpha$ and $G_{11}\alpha$, respectively [7,8].

All results described so far in this area were obtained under detergent-free conditions of cell fractionation. However, detergents may be used as very useful means how to distinguish among various forms of 'plasma membrane organisation' and these approaches have recently gained important implications in analysis of hormone action [11,12]. DRMs or sphingolipid-cholesterol membrane rafts, also known as DIMs (low-density detergent-insensitive or insoluble membranes), glycolipid-enriched membranes or detergent-insoluble glycolipid-enriched domains (DIGs) have been suggested to be involved in membrane trafficking, cell morphogenesis and signal transduction mechanisms [27,28]. Furthermore, caveolae, originally defined as flask-shaped invaginations of the plasma membrane, which are related to raft domains in terms of composition and/or topography [29,30], have been identified as important topological determinants of hormone or neurotransmitter action.

Association of $G_q\alpha/G_{11}\alpha$ proteins with caveolae was first demonstrated in MDCK canine kidney cells [19]. Subsequently, de Weerd and Leeb-Lundberg detected $G_q\alpha$ in caveolae of DDT_1 MF-2 smooth muscle cells where they observed a transient increase in $G_q\alpha$ content in caveolae induced by short-term treatment of cells with bradykinin [31]. Here, we report that $G_q\alpha/G_{11}\alpha$ proteins in E2M11-HEK cells are under resting conditions localised in a heterogeneous set of membrane domains (DIMs) exhibiting widely different densities, out of which caveolin-enriched domains represent only a minor portion. In addition, we show that sustained agonist treatment of these cells results in a dramatic redistribution of $G_q\alpha/G_{11}\alpha$ from DIMs to the bulk-membrane phase. These observations strongly support the notion about a role of DIMs in transmembrane signalling mediated by G

proteins and in the process of desensitisation of hormonal response.

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