Attenuation of $G_s\alpha$ coupling efficiency in brown-adipose-tissue plasma membranes from cold-acclimated hamsters

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In order to localize site(s) of β-adrenergic desensitization found in brown adipocytes from cold-acclimated animals, total brown-adipose-tissue homogenates (postnuclear supernatant) were obtained from control or cold-acclimated hamsters and were fractionated on discontinuous sucrose gradients. A low-density band (cytosolic proteins) and a high-density band (mitochondria) were obtained; in the middle fractions only low levels of protein were recovered. However, these fractions displayed a high level of specific [^{3}H]ouabain binding, indicating that they represented fractions enriched in plasma membranes. The level of [^{3}H]ouabain binding was significantly higher in plasma membranes from cold-acclimated animals, indicating an increased density of Na,K-ATPase units. The maximal activity of adenylate cyclase, as estimated with forskolin, was not changed by cold acclimation. However, the levels of cyclase activity observed after G_s-protein-mediated activation (with guanosine 5′-[γ-thio]triphosphate, isoprenaline, both of these, or fluoride) were decreased, indicating a decreased coupling efficiency. Notably, a significant decrease was observed in the functional activity of the G_s protein, as directly measured by estimation of the ability of cholate extracts of brown-fat plasma membranes to reconstitute G_s-protein-mediated stimulation of adenylate cyclase in cyc$^-$ membranes. Further, a functionally significant decrease (to 72%) was observed in the ratio between the amount of functional G_s proteins and adenylate cyclase units. The total content of $G_s\alpha$ protein was decreased to the same extent as the coupling efficiency of the membranes, indicating that a lower content of functionally equivalent $G_s\alpha$ molecules could explain the decreased coupling. It could therefore be concluded that a decrease in G_s-protein-mediated coupling efficiency, owing to a decrease in the amount of $G_s\alpha$, is at least one site of β-adrenergic desensitization in cold-acclimated animals. This may, at least in part, explain that desensitization takes place despite the fact that the β_3-adrenoceptor itself apparently lacks some of the sites known to be involved in the desensitization process in other β-adrenergic receptors.

INTRODUCTION

As a notable effect of cold-acclimation [1], a functional desensitization of β-adrenergic responses in brown adipocytes occurs. This is observable as a nearly 10-fold increase in the concentration of noradrenaline necessary for half-maximal stimulation of thermogenesis in isolated cells [2–5]. That a desensitization occurs due to the chronic noradrenaline stimulation of the brown-fat cells which takes place during cold-acclimation is not in itself unexpected. Such a desensitization process seems to develop as a consequence of sustained stimulation of any target tissue. The question is at which step in the signal-transmission pathway the desensitization takes place. Here the situation in brown adipose tissue may be somewhat special. This is because the β_3-adrenergic receptor (which is the one involved in the thermogenic response [6]) apparently lacks the phosphorylation sites which are found on other adrenergic receptors [7–9] and which are responsible for certain types of desensitization [10,11]. Thus, potential desensitization sites may probably be located at a later point in the transmission process; two candidate points would be the adenylate cyclase (EC 4.6.1.1) itself and the G-proteins which transduce the receptor signals to the cyclase.

To allow for an identification of the site(s) involved in the desensitization process, we have here prepared purified plasma membranes from brown adipose tissue, and we have used these membranes to investigate mediation of β-adrenergic stimulation, i.e. adenylate cyclase activation. As receptor control of adenylate cyclase is mediated via G-proteins [12–14], we have investigated the functional activity and the specific content of $G_s\alpha$ (α-subunit of stimulatory G-protein) in brown-adipose-tissue plasma membranes. For this, we have used the adenylate cyclase complementation assay introduced by Gilman and co-workers [12,15], as well as immunoblot analysis with anti-$G_s\alpha$-peptide antibodies. We found a significant decrease in the amount of functional G_s protein per unit of adenylate cyclase in the plasma membranes of cold-acclimated animals; this decrease was paralleled by a decrease in $G_s\alpha$ protein. These results may, at least in part, explain the cold-induced desensitization.

MATERIALS AND METHODS

Subcellular fractionation of brown-adipose-tissue homogenate

Adult golden hamsters (*Mesocricetus auratus*) were kept at +20 °C (control) or at +4 °C (cold-acclimated) for at least 4 weeks with free access to food and water. Fractionations and all other procedures were always performed with parallel preparations from cold-acclimated and control hamsters. The animals were killed by CO_2, followed by decapitation, and the brown adipose tissue was removed and minced with scissors in 0.25 M sucrose/20 mM Tris/HCl (pH 7.4)/1 mM EDTA. Homogenization was performed on ice with a Potter–Elvehjem homogenizer with a tightly fitting Teflon pestle (20–30 strokes). In the standard isolation procedure, the pooled brown adipose tissue obtained from 4 (control) or 2–4 (cold-acclimated) animals was homogenized separately in identical volumes of sucrose

Abbreviations used: G_s, the stimulatory G-protein; G_i, the inhibitory G-protein; $G_s\alpha$, the α subunit of G_s; GTP[S], guanosine 5′-[γ-thio]triphosphate.

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medium (1–2 g wet wt. of tissue/20 ml) and was then kept on ice for 10–15 min to compact the fat; it was then filtered through a double layer of silk cloth and centrifuged at 600 *g* for 5 min. The resulting supernatant (essentially free of fat and nuclear debris) was divided into 4+8+8 ml portions. The 4 ml volume of the 600 *g* supernatants was diluted 1:1 with sucrose medium and centrifuged for 60 min at 150000 *g*; the resulting pellets (crude membranes) were resuspended in 20 mM Tris/HCl (pH 7.4)/1 mM EDTA and stored at −80 °C. The two volumes of 8 ml each were applied to the top of two discontinuous sucrose-density gradients consisting of 19, 23, 27, 31, 35, and 43% (g/100 g) sucrose in 20 mM Hepes (pH 8.0)/1 mM EDTA (5 ml per sucrose layer). This type of gradient was essentially the same as that described previously for isolation of plasma membranes from S49 lymphoma cells [16,17]. The gradients were centrifuged for 60 min at 27000 rev./min (85000 *g*) in a Beckman SW 28 rotor at 4 °C. The compact layer of residual fat was removed manually from the meniscus. The first 7 ml fraction represented the overlaid medium (fraction 0), and the rest of the gradient was collected in 5 ml fractions; fraction 1 was thus composed of 1 ml of overlay plus 4 ml of sucrose of the lowest density (19%) etc. The corresponding gradient fractions from the two identical gradients were pooled, diluted with 20 ml of 20 mM Tris/HCl (pH 8.0)/1 mM EDTA and centrifuged for 60 min at 150000 *g*. The final membrane sediments were resuspended by manual homogenization (Teflon pestle) and pipetting and were stored at a concentration of 5–15 mg of membrane protein/ml in 20 mM Tris/HCl (pH 7.4)/1 mM EDTA at −80 °C.

Protein was determined by the Lowry method, with BSA as standard.

Isolation of plasma membranes from isolated brown-fat cells

Brown-fat cells were isolated by the collagenase method as previously described [4]. The final cell suspension prepared from 6 (control) or 2 (cold-acclimated) hamsters was resuspended in 10 ml of Krebs–Ringer phosphate buffer, frozen in liquid nitrogen and stored at −80 °C. Homogenization of the thawed cell suspension and all subsequent steps of purification of plasma membranes were carried out principally as described above for tissue homogenate.

Measurement of ouabain binding

The amount of Na,K-ATPase (EC 3.6.1.37; used as a plasma-membrane marker) was quantified by measurement of the binding capacity for the radioligand [^{3}H]ouabain, essentially as described previously [18]. Portions of brown-adipose-tissue membrane suspensions were incubated with [^{3}H]ouabain in a total volume of 0.4 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 started by addition of [^{3}H]ouabain and continued for 60 min. In saturation-binding experiments, the membrane fractions were incubated with increasing concentrations of [^{3}H]ouabain, 0.03–280 nM. When the number of specific [^{3}H]ouabain-binding sites was compared among different density gradient fractions ('one-point assays'), a concentration of 140 nM [^{3}H]ouabain was used. In all cases, the incubations were terminated by dilution with 5 ml of ice-cold incubation buffer and filtration through Whatman GFC filters. The filters were washed with 2 × 5 ml of cold buffer, dried overnight at room temperature, and the radioactivity was determined by liquid-scintillation counting. Non-specific binding was determined in the presence of 10 μM unlabelled ouabain. Non-linear regression analysis of the saturation-binding data was performed by fitting the data to a rectangular hyperbola (binding isotherm), $Y = B_{max.} \cdot X/(K_d + X)$, where $B_{max.}$ is the maximum binding capacity, K_d is the dissociation constant and X is the free concentration of radioligand (GraphPad). The binding data were also analysed in accordance with Scatchard.

Assay of adenylate cyclase activity

Adenylate cyclase activity was determined in the membrane fractions by the method of Salomon et al. [19], which involves the separation of cyclic [^{32}P]AMP from [^{32}P]ATP on sequential columns of Dowex 50 and alumina. Briefly, the membrane fractions (10–50 μg of protein per assay) were 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 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 [^{32}P]ATP (about 1000000 c.p.m. per sample). The activating ligands fluoride (13.3 μM $AlCl_3$ + 6.6 mM NaF + 10 mM $MgCl_2$), guanosine, 5′-[γ-thio]triphosphate (GTP[S] (20 μM), isoprenaline (10 μM) or forskolin (50 μM) 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 (20000 c.p.m. per assay as internal standard) and heating for 5 min at 95 °C.

Assay of functional activity of $G_s\alpha$

The functional activity of $G_s\alpha$ was determined by a complementation assay *in vitro* utilizing the mutant S49 lymphoma cell line, cyc$^-$, which is devoid of $G_s\alpha$ [12,15]. The brown-adipose-tissue plasma-membrane fractions were extracted with 1% sodium cholate (0 °C, 60 min, with vortex-mixing every 5 min) and centrifuged for 30 min at 100000 *g*. The 100000 *g* supernatants (the cholate extracts containing the G-proteins; 9 μl, 10–30 μg of protein) were incubated with cyc$^-$ mutant membranes (10 μl, 40–80 μg of protein; prepared as described by Ross et al. [15]). Then the adenylate cyclase enzyme reaction was started by addition of [^{32}P]ATP (40–100 c.p.m./pmol) and activators as indicated and discontinued after 30 min by addition of the stop solution described above and heating for 5 min at 95 °C. The adenylate cyclase activity of brown-adipose-tissue cholate extracts was zero. The basal adenylate cyclase activity, measured with or without NaF, in the cyc$^-$ mutant membranes alone was less than 5% of the G_s-reconstituted level and was subtracted from the total enzyme activity.

Assay of $G_s\alpha$ content

The content of $G_s\alpha$ was determined by immunoblotting techniques using an antiserum against a specific $G_s\alpha$ peptide [20,21]. The antibody was raised to the peptide TPEPGEDPRVTR (amino acids 325–336 of $G_s\alpha$).

The proteins of the brown-adipose-tissue plasma-membrane fractions were resolved by SDS/PAGE in 12% acrylamide gels with discontinuous buffer systems as described in [22]. The separated proteins were electrophoretically transferred to nitrocellulose membranes (0.45 μm pore size). To block unspecific binding, the membranes were preincubated with 4% BSA in Tris-buffered saline for 1 h. They were then incubated for 2 h with the rabbit anti-($G_s\alpha$ peptide) polyclonal antiserum diluted 1:2000. The $G_s\alpha$ protein bands were revealed with alkaline-phosphatase-conjugated goat anti-rabbit IgG, diluted 1:100000 (Sigma). The resulting spots were scanned with a densitometric scanner.

Chemicals

[21,22-^{3}H]Ouabain (TRK.429; 1.33 TBq/mmol, 36 Ci/mmol), [α-^{32}P]ATP (PB.160; 15 TBq/mmol, 400 Ci/mmol) and [2,8-^{3}H]adenosine 3′,5′-cyclic monophosphate were purchased from Amersham. Forskolin (F6886) and ouabain (A9501) were obtained from Sigma. Sucrose was of AristaR grade, from BDH. Cys-Thr-Pro-Glu-Pro-Gly-Glu-Asp-Pro-Arg-Val-Thr-Arg, i.e. the N-terminal cysteine-extended peptide fragment 325–336 (TPEPGEDPRVTR) of the $G_s\alpha$ protein, was purchased from Gramsch Laboratories, Schwabhausen, Germany.

RESULTS

Site(s) involved in the adrenergic desensitization process are likely to be found in the plasma membrane. We used a previously developed method for preparation of purified plasma membranes to obtain such membranes from brown adipose tissue, and we used [^{3}H]ouabain binding to confirm the localization of the plasma-membrane fractions. These membranes, obtained from control and cold-acclimated animals, were then used to identify sites of desensitization during the mediation of β-adrenergic stimulation; the activity of the catalytic unit of adenylate cyclase, the mediation of signals via the G_s protein and the total content of $G_s\alpha$ were analysed.

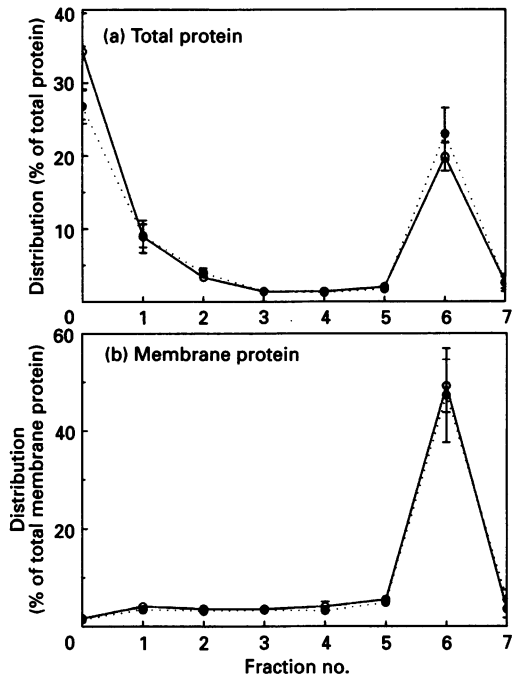

Figure 1 Distribution of (a) total protein and (b) membrane protein on the sucrose density gradients

(**a**) Tissue homogenates (see the Materials and methods section) from control or cold-acclimated animals were applied to the discontinuous sucrose-density gradients and fractionated as described in the Materials and methods section. Protein was determined directly in the sucrose fractions. The results are expressed as percentage of the protein amount found in the 600 *g* supernatant applied to the gradient. The total amount of protein (100%) present in the 600 *g* supernatant represented 78 ± 9 mg per control hamster and 152 ± 21 mg per cold-acclimated hamster, but the amount of protein actually applied was practically identical in the parallel fractionation procedures (control 312 ± 36 mg and cold-acclimated 304 ± 36 mg). The values shown are means ± S.E.M. from five separate experiments (fractionations). ○, Preparations from control hamsters; ●, preparations from cold-acclimated hamsters. (**b**) The sucrose-density-gradient fractions from (**a**) were diluted and centrifuged as described in the Materials and methods section, and the amount of protein was determined in the membrane pellets. The total (100%) amount of membrane protein applied to the fractionation was defined as that found in the crude membranes which were sedimented from the 600 *g* supernatant by centrifugation at 150000 *g* (see the Materials and methods section); this amount was 30 ± 4 mg for control and 60 ± 8 mg for cold-acclimated hamsters, expressed per animal. The values shown are thus the recovery of membrane protein, and represent means ± S.E.M. from the five separate experiments (fractionations).

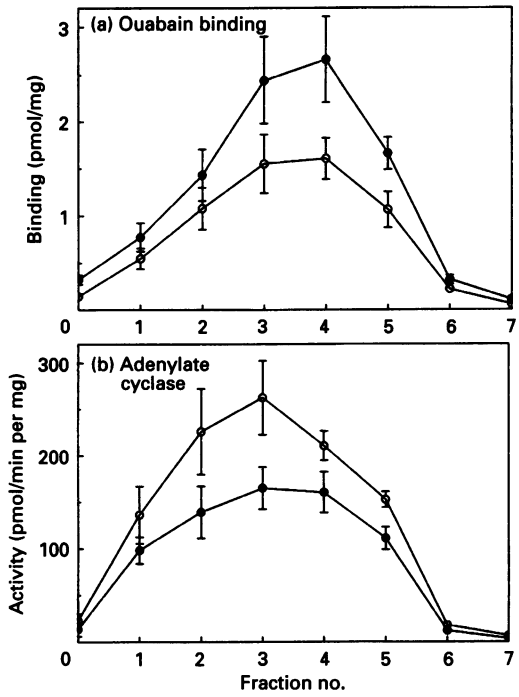

Figure 2 Distribution of plasma-membrane markers on the sucrose density gradients: (a) specific [^{3}H]ouabain binding; (b) fluoride-stimulated adenylate cyclase

(**a**) The membrane proteins from fractions 0–7 (see Figure 1b) (50–300 μg per sample) were incubated with 140 nM [^{3}H]ouabain, and the specific binding was determined as described in the Materials and methods section. Non-specific binding was less than 10% of the total binding. The data shown are means ± S.E.M. from four fractionation procedures, each determined at least in duplicate. ○, Preparations from control hamsters; ●, preparations from cold-acclimated hamsters. (**b**) Fluoride-stimulated adenylate cyclase activity (see the Materials and methods section) was determined in the gradient fractions 0–7. The data shown are means ± S.E.M. from three fractionation procedures, each determined in duplicate.

Subcellular fractionation of brown adipose tissue

After fractionation of total brown-adipose-tissue homogenates on sucrose-density gradients with the discontinuous gradient method [16,17], two major protein bands could be distinguished (Figure 1a). The first band appeared at the low-density end of the gradient (close to the meniscus) in fractions 0 and 1; the second protein band hovered over the bottom of the centrifuge tube in fraction 6. The gradient fractions between these two dominant bands, i.e. fractions 2–5, contained only little protein, and there was no sign of any minor local peak in this region. Thus, on the basis of protein distribution, three main areas of the density gradient were found: a low-density band, a middle portion and a high-density band. From a comparison between the gradient profiles of total protein (Figure 1a) and membrane protein (Figure 1b), it is apparent that the low-density band mainly consisted of cytosolic proteins; the other gradient fractions, i.e. fractions 2–7, consisted practically entirely of membrane protein. On the basis of analyses performed previously with this fractionation method [17], it is likely that the high-density band in fraction 6 is mainly composed of mitochondria.

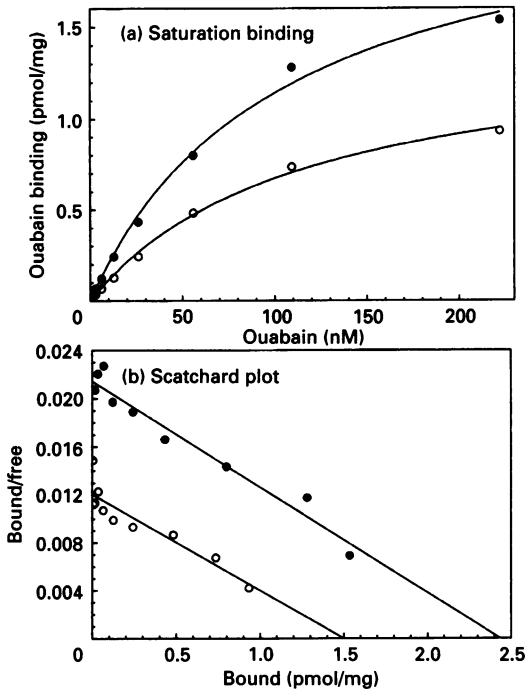

Figure 3 (a) Saturation binding curves for [^{3}H]ouabain binding to fractions 3+4 (plasma membranes) from cold-acclimated and control hamsters; (b) Scatchard plot of the binding data

The data shown are from one fractionation of membranes from cold-acclimated and control animals (140 μg protein per assay). The K_d and $B_{max.}$ values were calculated both by direct fitting to a saturation binding curve (rectangular hyperbola) with the help of the GraphPad program (**a**) and by Scatchard-plot analysis (**b**). Both methods of calculation yielded virtually the same results: K_d values of 113 and 100 nM, and $B_{max.}$ values of 1.5 and 2.4 pmol/mg, for control and cold-acclimated samples respectively.

The membrane protein (Figure 1b) represented about 50% of total protein (Figure 1a). There was no notable difference in total protein distribution or membrane protein distribution between preparations from control and cold-acclimated animals.

Ouabain binding

To confirm the localization of the plasma-membrane fractions, the amount of specific [^{3}H]ouabain binding (used as a measurement of the number of units of Na,K-ATPase) was used as a marker. The peak values for specific binding capacity were obtained in fractions 3–4 (Figure 2a). The values were about 15 times higher in the peak fractions than in the crude membrane preparations; this indicated a considerable purification of plasma membrane in these fractions, and it may therefore be concluded that the lower–middle portion of the density gradient, above the heavy protein band, contained enriched plasma-membrane fragments.

The nature of the [^{3}H]ouabain binding was further investigated in saturation-binding studies performed with some of the plasma-membrane fractions. As exemplified in Figure 3, the [^{3}H]ouabain binding was saturable (Figure 3a), and computer analysis of the binding data (GraphPad) revealed a single set of binding sites. The affinity of the ouabain-binding site was not changed by cold-acclimation, but the binding capacity was increased. In agreement with this, the average [^{3}H]ouabain-binding capacity in the gradient fractions 0–6 prepared from cold-acclimated hamsters was consistently higher than in those from the control membranes (Figure 2a). Comparison of the two groups by Student's paired t test, each pair represented by a given fraction measured in a given experiment, showed a highly significant increase ($P < 0.001$). Thus it would seem that cold-acclimation led to a markedly increased density of Na,K-ATPase units in brown-fat plasma membranes. It may be added that, due to the mitochondrial proliferation during cold acclimation, any mitochondrial-membrane contamination of the plasma-membrane fraction would lead to a decrease in the density of [^{3}H]ouabain-binding sites; that the density in fact increased is in itself an indication that the plasma-membrane fraction studied is free from mitochondrial contaminants.

Adenylate cyclase

In order to delineate the mechanism of decreased sensitivity to noradrenaline seen in brown adipocytes from cold-acclimated animals, we used the gradient fractions described above to examine the activity of adenylate cyclase, both the catalytic unit itself (stimulation by forskolin) and the activity when stimulated via G_s-proteins (GTP[S], isoprenaline+GTP[S], fluoride). Specific adenylate cyclase activity reached a peak in the same fractions as [^{3}H]ouabain binding (Figure 2b). For the following studies, fractions 3–5 were combined.

In order to measure the maximum catalytical activity of the adenylate cyclase, the forskolin-stimulated activity was investigated. As shown in Table 1, a high activity was observed, but no significant difference was found between the two groups of animals investigated, indicating that the total complement of adenylate cyclase enzyme per plasma-membrane unit was not changed by cold-acclimation.

When the adenylate cyclase was stimulated via a G-protein-mediated pathway, by GTP[S] alone, by isoprenaline, by isoprenaline+GTP[S], or by fluoride, the activities measured were lower than those induced by forskolin, indicating that the functional activity of the G_s-proteins was rate-limiting (Table 1). Further, it was very noteworthy that, whenever the adenylate cyclase was stimulated in this way via a G_s-protein-mediated pathway, a consistent and significantly lower adenylate cyclase activity was observed in the plasma membranes from cold-acclimated animals than in membranes from controls. The consistently decreased activity of adenylate cyclase observed in preparations from cold-acclimated hamsters with G_s-protein-mediated activators indicated that the functional activity of the G_s-protein was decreased in plasma membranes prepared from cold-acclimated hamsters.

This conclusion was further supported by preliminary results obtained on plasma-membrane fractions prepared from brown-fat cells. The isoprenaline+GTP[S]-stimulated adenylate cyclase was decreased to 55% and the fluoride-stimulated activity to 68%, as compared with control levels. (Low protein yields precluded extensive studies using these membranes.)

Reconstitution assay

From the indications noted above of a decreased adenylate cyclase activity in membranes from cold-acclimated animals when stimulation was mediated via G_s-protein, it was decided to measure directly the coupling activity of G_s-proteins in the different plasma membranes. For this purpose, the adenylate cyclase complementation assay introduced by Sternweis and Gilman [12] was used. This assay is based on the ability of detergent extracts of plasma membranes to reconstitute the G_s-mediated stimulation of adenylate cyclase activity in cyc$^-$ mutant cells. The cyc$^-$ mutant of the S49 lymphoma cell line lacks the

Table 1 Efficiency of different agents to stimulate adenylate cyclase activity in brown-adipose-tissue plasma membranes

Membranes from fractions 3, 4 and 5 were collected from the gradients and adenylate cyclase activity was measured as described in the Materials and methods section, in the presence of the indicated agents: forskolin 50 μM; isoprenaline 10 μM; GTP[S] 20 μM; fluoride = 13.3 μM $AlCl_3$ + 6.6 mM NaF + 10 mM $MgCl_2$. Adenylate cyclase activities are presented as pmol of cyclic AMP/min per mg of brown-fat plasma-membrane protein. Results are means ± S.E.M. from four fractionation procedures (basal and isoprenaline are from one fractionation series; the relatively high isoprenaline-stimulated enzyme activity may indicate the presence of endogenous guanine nucleotides, and further characterization was not meaningful): *indicates a significant difference between membranes from control and cold-acclimated animals ($P < 0.05$; Student's paired t test on the normalized values); N.S., not significant ($P > 0.05$).

	Control	Cold	Cold/control (%)
Basal	9	8	–
Forskolin	382 ± 59	320 ± 49	85 ± 8^{NS}
Isoprenaline	137	76	55
Isoprenaline + GTP[S]	281 ± 15	168 ± 21	60 ± 8*
GTP[S]	170 ± 20	107 ± 26	61 ± 11*
Fluoride	169 ± 22	135 ± 28	78 ± 9

Table 2 Efficiency of different agents to stimulate adenylate cyclase in cyc$^-$ membranes after reconstitution with cholate extracts of brown-adipose-tissue plasma membranes

Reconstitution of G_s-mediated adenylate cyclase activity in cyc$^-$ mutant membranes with cholate extracts of brown-fat plasma membranes was performed as described in the Materials and methods section. The reconstituted activities are presented as pmol of cyclic AMP/min per mg of brown-fat plasma-membrane protein originally used for the cholate extraction. The concentrations of agents were the same as those in Table 1. The values are means ± S.E.M. from three independent fractionation procedures, each assayed in duplicate (the fluoride values are from five independent fractionation procedures, each assayed 2–8 times). * indicates a significant effect of cold-acclimation ($P < 0.05$; Student's paired t test on the normalized values).

	Control	Cold	Cold/control (%)
Basal	2 ± 1	1 ± 1	–
Isoprenaline	6 ± 1	5 ± 1	–
Isoprenaline + GTP[S]	71 ± 2	52 ± 2	73 ± 5*
GTP[S]	62 ± 3	48 ± 3	78 ± 4*
Fluoride	62 ± 11	41 ± 11	66 ± 9*

Table 3 Ratio between the amount of functional G_s protein and adenylate cyclase activity in plasma-membrane fractions from control and cold-acclimated hamsters

The values are based on measurements of forskolin-stimulated adenylate cyclase activity (see Table 1) and of fluoride-stimulated reconstitutive G_s activity (see Table 2) in each of four fractionation procedures and represent the ratio between these two activities: ** indicates a significant effect of cold-acclimation ($P < 0.01$; Student's paired t test on the normalized values).

	Ratio adenylate cyclase/G_s
Control	0.21 ± 0.09
Cold	0.16 ± 0.07
Control/cold	0.72 ± 0.06**

$G_s\alpha$ subunit. Fusion of exogenous $G_s\alpha$ (in detergent extracts) with the cyc$^-$ membranes restores the $G_s\alpha$-mediated stimulation of cyc$^-$ adenylate cyclase.

The gradient fractions 3–5 (the plasma membranes) were extracted with cholate and the adenylate cyclase complementation assay was performed. In the assay, the adenylate cyclase activity of the cyc$^-$ mutant membranes, or of cholate extracts alone, was less than 1 pmol/min per mg, and the reconstituted levels without addition of activators were also very low (Table 2). Addition of isoprenaline alone led only to a small increase in adenylate cyclase activity. However, a marked stimulation of adenylate cyclase activity was observed in the presence of GTP[S], of isoprenaline + GTP[S], or of fluoride, indicating that the reconstitution was successful (Table 2).

It was very clear from a comparison of the two experimental groups that, independent of which method for estimation of coupling activity was used, there was a significant decrease in reconstitution potential in the plasma membranes of cold-acclimated animals. Thus, functional G_s activity was clearly decreased in these membranes.

Amount of functional G_s protein per adenylate cyclase

In order to verify that cold-acclimation led to a decrease in the amount of functional G_s protein per unit of adenylate cyclase activity, we have tabulated the ratio between the functional G_s activity (measured as reconstituted fluoride-stimulated adenylate cyclase activity in cyc$^-$ membranes) and the total adenylate cyclase activity in that fraction (measured as forskolin-stimulated adenylate cyclase activity) (Table 3). Through this procedure, any problems resulting from different purities of the plasma-membrane fractions in different fractionation procedures are eliminated.

Table 3 shows that a consistent decrease in the ratio between functional G_s activity and functional adenylate cyclase activity was found in the preparations from cold-acclimated animals. Assuming that the amount of functional G_s protein may be rate-limiting in the transfer of information from the β-adrenergic receptor to the cyclase, this result would indicate that this information transfer would be hampered in the cold-acclimated animals.

$G_s\alpha$ content

In order to investigate whether the decrease in coupling activity was due to a decrease in the amount of $G_s\alpha$ protein or to a modification of $G_s\alpha$ activity, the content of $G_s\alpha$ protein in plasma membranes of brown adipose tissue was investigated by an immunoblot method. Plasma-membrane proteins were resolved by SDS/PAGE, and the amount of $G_s\alpha$ protein was quantified by immunoblot analysis with specific anti-($G_s\alpha$ peptide) antiserum (Table 4). There was a clear effect of cold-acclimation on the content of $G_s\alpha$: a decrease to 79% of the control value.

In parallel with the determination of the content of $G_s\alpha$ in these preparations, their content of adenylate cyclase and the functional activity of the $G_s\alpha$ were also determined as described previously (Table 4). In agreement with our observations above, the content of (forskolin-stimulated) adenylyl cyclase was unchanged by cold-acclimation, whereas the isoprenaline + GTP[S]-stimulated adenylate cyclase was decreased to 76%. Similarly, the reconstitution activity was decreased to 83%. The coupling ratio (reconstitution/adenylyl cyclase) was decreased to 76%, confirming the attenuation of coupling in the plasma membranes from cold-acclimated animals.

Thus the reconstitution activity was decreased to 83%, and the content of $G_s\alpha$ proteins to 79%, of the control values. From the similarity of these values, it could be concluded that the

Table 4 Effect of cold-acclimation on $G_s\alpha$ content and activity

The data are means ± S.E.M. from 6 independent plasma-membrane preparations (i.e. fractions 3+4), each analysed in duplicate. The specific adenylate cyclase or reconstitutive activity is expressed in pmol of cyclic AMP/min per mg. The reconstitutive/forskolin value represents the ratio between the functional G_s protein and the maximum adenylate cyclase activity in the plasma-membrane fractions from control and cold-acclimated hamsters. For determinations of the amount of $G_s\alpha$ protein, 60–98 μg of membrane protein (the same amount for each parallel membrane preparation) was applied per electrophoretic lane, and the total amount of $G_s\alpha$ protein was analysed by the immunoblot method described in the Materials and methods, the result of which was quantified densitometrically: *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) indicate significant effects of cold-acclimation (Student's paired t test on the normalized values); NS, not significant.

	Control	Cold	Cold/control (%)
Adenylate cyclase			
Forskolin-stimulated	293 ± 35	313 ± 52	107 ± 10^{NS}
Isoprenaline + GTP[S]	235 ± 23	177 ± 8	76 ± 7*
Reconstitutive activity			
Fluoride-stimulated	86 ± 5	71 ± 5	83 ± 3**
Reconstitutive/forskolin	0.30 ± 0.04	0.23 ± 0.05	76 ± 7*
$G_s\alpha$ protein			
$G_s\alpha$ (relative amount)	100	79 ± 4	79 ± 4***

functional attenuation of coupling activity in brown-adipose-tissue membranes, seen after cold-acclimation, was due to a decrease in the amount of $G_s\alpha$ protein.

DISCUSSION

In the present investigation, we have fractionated brown adipose tissue to obtain plasma membranes. With these membranes we have observed that cold-acclimation led to an increased level of [^{3}H]ouabain binding in the plasma membranes, but the level of adenylate cyclase was unchanged. However, there was a consistent decrease both in the functional activity and in the amount of $G_s\alpha$ in the plasma membranes from cold-acclimated animals, and this could be at least one of the factors which lead to the adrenergic desensitization observed in brown-fat cells isolated from cold-acclimated animals.

Plasma membrane

In order to obtain plasma-membrane fractions from brown adipose tissue, we have used a method for subcellular fractionation, developed in other systems [16,17]. Compared with methods used previously for subcellular fractionation of brown adipose tissue [23–25], the technical difference from the present method is primarily that an initial differential-centrifugation procedure (or a series of high-speed centrifugations) is not used; rather, the complete postnuclear homogenate is the initial material for the fractionation, and a fraction of the plasma membranes of the tissue is therefore not initially discarded. Further, the risk of a loss of components only loosely associated with the plasma membranes [26,27], or of inactivation of labile enzymes such as adenylate cyclase, is probably diminished.

Cold-induced increase in Na,K-ATPase density

The presence of specific [^{3}H]ouabain-binding site was used here as a marker for the plasma membrane. The amount of specific ouabain binding is expected to reflect the amount of Na,K-ATPase units (although it cannot be fully excluded that alterations in, e.g., membrane lipid composition, may alter the binding characteristics of the [^{3}H]ouabain-binding site of the Na,K-ATPase). As an effect of cold-acclimation, a notable increase in the amount of [^{3}H]ouabain-binding sites in the plasma membranes was observed (Figure 2), presumably reflecting an increase in the amount of Na,K-ATPase.

Although the Na,K-ATPase was originally suggested to play a significant role in the thermogenic process [28,29], the idea of such a role for this enzyme is no longer maintained. However, it has been established that a small fraction of total thermogenesis (about 5–10%) may originate from Na,K-ATPase activity [30], and during noradrenaline-induced thermogenesis it is likely that a sustained increase in influx of Na^+ [31] and outflux of K^+ [32] is occurring. This could well increase the demand for Na,K-ATPase activity, and a compensatory increase in the amount of Na,K-ATPase units may occur.

Localization of the adrenergic desensitization event

That cold-acclimation is associated with a decrease in noradrenaline affinity in brown adipose tissue is well known. Such a decrease in sensitivity must have a molecular explanation. Potential sites of desensitization would be the content of β_3-receptors, receptor modification or intracellular sequestration of receptors. The desensitization sites could also be found at the post-receptor level, i.e. at the adenylate cyclase level, or in the interaction between the receptor and the cyclase, i.e. at the G-protein level. It is not unlikely that desensitization events may take place at several of these levels. Upon short-term cold exposure, there is indeed evidence for a decreased amount of mRNA for β_3-receptors [33]. Further, although there are technical problems in detecting accurately the amount of β_3-receptors (there is no high-affinity selective ligand available), reports exist of a decreased β_3-receptor density upon chronic pharmacological β_3-stimulation [34]. In the present investigation, however, we have particularly studied the post-receptor events, primarily the adenylate cyclase and the coupling between the β_3-receptors and the cyclase.

An unchanged adenylate cyclase activity

Adenylate cyclase mediates the action of noradrenaline, by elevating the cytosolic cyclic AMP concentrations, and this then stimulates lipolysis and ultimately thermogenesis in the brown adipocytes [35]. It could therefore be considered likely that the observed adrenergic desensitization could be due to a decrease in adenylate cyclase activity. However, when the adenylate cyclase was stimulated by the diterpene forskolin, which activates the catalytical unit directly [36], no significant difference was observed in the activity in membranes from cold-acclimated or control animals (Tables 1 and 4), and the adenylate cyclase is therefore an unlikely desensitization site.

A decrease in functional G-protein

As the receptor-mediated control of adenylate cyclase activity is elicited through the G-proteins, the desensitization process may take place at the level of the G-proteins. When we assessed the functional activity of the G_s protein by a reconstitution assay, we observed that the cholate extracts of brown-adipose-tissue membranes prepared from cold-acclimated animals were significantly less potent in reconstituting receptor/cyclase coupling than were extracts from control samples (Tables 2–4).

Such a mechanism for a desensitization process is not without precedence, at least not in studies *in vitro*. Thus, isoprenaline stimulation of S49 lymphoma cells leads to a decrease in the plasma-membrane levels of the $G_s\alpha$ subunit (and a corresponding increase in the cytosol) [27], and prostaglandin E1 stimulation of

neuroblastoma–glioma cell lines also leads to a decrease in $G_s\alpha$ levels [37]. Similarly, prolonged adenosine stimulation of white-fat cells leads to a decrease in all three (G_i1, G_i2 and G_i3) types of $G_i\alpha$ proteins [38,39], in agreement with the fact that the adenosine A1 receptor acts via G_i proteins. What is of special interest in the present study is thus the fact that such a decrease in G-protein functional activity can be demonstrated to take place during a physiological desensitization process, occurring within the animal as a response to a continued physiological stimulus.

It should be noted that this decrease in functional G_s activity during prolonged adrenergic stimulation is quite another phenomenon than the increase in (functional) G_s amount (as estimated by various parameters) which has been well documented by Granneman and co-workers [25,40–45] during the transition phase, which leads to a recruited state in the tissue, e.g. during the first days of a cold stress which with time leads to the fully cold-acclimated state investigated here.

Molecular basis for the attenuated coupling efficiency

The attenuated coupling efficiency of plasma membranes from cold-acclimated animals was parallelled by alterations in the content of $G_s\alpha$ protein in the membranes (Table 4). Quantitatively, there was a very similar decrease in the functional activity (to 83%) and in the total content of $G_s\alpha$ (to 79%). It therefore seems that the attenuated coupling efficiency could be fully explained by a decrease in the amount of $G_s\alpha$, and that alterations in the coupling efficiency of individual $G_s\alpha$ molecules need not be evoked.

It is noteworthy that, in a perinatal investigation in brown adipose tissue, changes in $G_s\alpha$ content have been observed previously: the level of $G_s\alpha$ was decreased during the first week after birth (relative to the level shortly before birth), but then slowly increased during the following weeks [43,44]. Although this perinatal pattern could perhaps be understood as an innate ontogenically governed pattern, it is more likely that the response is related to the altered thermic demands on the pup, i.e. the brown adipose tissue is in a recruited state during the first week after birth and thereafter atrophies [46,47]. With this interpretation of the perinatal changes in brown adipose tissue, both the perinatal change in the content of $G_s\alpha$, and the change reported here due to cold-acclimation, adhere to a general pattern of decreased levels of $G_s\alpha$ in functionally recruited states of brown adipose tissue.

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