The decrease in the short variant of $G_s\alpha$ protein is associated with an increase in [3H]CGP12177 binding, [3H]ouabain binding and Na,K-ATPase activity in brown adipose tissue plasma membranes of cold-acclimated hamsters

L Bourová, J Novotný and P Svoboda

Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Praha 4, Czech Republic

(Requests for offprints should be addressed to P Svoboda)

ABSTRACT

Sucrose density gradient purified plasma membranes isolated from brown adipose tissue of cold-acclimated hamsters (4–10 weeks at 0–4 °C) were analysed for the content of the short ($G_s\alpha S$) and long ($G_s\alpha L$) variants of $G_s\alpha$ protein (the α subunit of the stimulatory G protein) and compared with the membranes isolated from control animals. The relative ratio between the two variants ($G_s\alpha S/G_s\alpha L$) decreased from 0.48 to 0.24 ($P < 0.01$). This result, obtained by electrophoretic resolution of membrane proteins by standard SDS-PAGE and an immunoblot analysis with an antiserum oriented against an internal sequence of $G_s\alpha$, was verified by resolution on urea-containing gels and an antiserum oriented against the C-terminus decapeptide of $G_s\alpha$.

Under these conditions, the $G_s\alpha S/G_s\alpha L$ ratio was decreased from 0.41 to 0.31 ($P < 0.05$). The total amount of both isoforms ($G_s\alpha S$ plus $G_s\alpha L$) decreased to 83% ($P < 0.05$) or 68% ($P < 0.01$) by standard or urea SDS-PAGE respectively. These data demonstrate that cold-acclimation of hamster brown adipose tissue is associated with preferential decrease in the plasma membrane density of the short variant of the $G_s\alpha$ protein.

This decrease was paralleled by an increase in the other plasma membrane constituents, [3H]CGP12177 binding sites, [3H]ouabain binding sites and Na,K-ATPase activity to 147%, 212% and 191% respectively.

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INTRODUCTION

In studies of desensitisation of β -adrenergic hormone response (Benovic *et al.* 1988, Hausdorff *et al.* 1990, Kobilka 1992, Collins 1993, Lohse 1993), brown adipose tissue provides an interesting opportunity to study the long-term type of this phenomenon under *in vivo* conditions. This is because brown adipose tissue is constantly stimulated by norepinephrine when the animal is exposed to cold (Feist 1970, Sigurdson & Himms-Hagen 1988). The cells (Nedergaard 1982, Svartengren *et al.* 1982, Unnelius 1990, 1993), tissue pieces (Freidli *et al.* 1978, Kuroshima *et al.* 1991), crude membranes (Svartengren *et al.* 1984) or plasma membrane-enriched fractions (Svoboda *et al.* 1984a, 1993) prepared from brown adipose tissue of cold-acclimated animals have a decreased respiratory

(Nedergaard 1982, Svartengren *et al.* 1982, Unnelius 1990, 1993), cAMP (Svartengren *et al.* 1982), adenylyl cyclase (Svartengren *et al.* 1984, Svoboda *et al.* 1984a) or thermogenic (Freidli *et al.* 1978) response to β -adrenergic stimulation.

Previously, we have investigated different aspects of this physiologically induced desensitisation and have presented evidence that the functional activity (adenylyl cyclase activity and cyc^- reconstitution assays) as well as the total amount of $G_s\alpha$ protein (the α subunit of the stimulatory G protein) were decreased (Svoboda *et al.* 1993). We have also tested another possibility, namely, that the influence elicited by inhibitory G proteins (G_i proteins) on the stimulatory arm of the norepinephrine cascade would be altered by cold acclimation (Svoboda *et al.* 1996). Contrary to our original expectations we found that both functionally and in content,

the amount of G_i was decreased due to cold acclimation.

In this work, the individual isoforms (variants) of $G_s\alpha$ protein were resolved and analysed by SDS-PAGE and immunoblotting of density gradient purified plasma membranes. The membrane density of $G_s\alpha$ protein levels was compared with the density of the other plasma membrane constituents, [^3H]CGP12177 binding sites, [^3H]ouabain binding sites and Na,K-ATPase activity.

MATERIALS AND METHODS

Materials

(-)-[^3H]CGP12177 (TRK 835; 1.67 TBq/mmol, 45 Ci/mmol) and [21,22- ^3H]ouabain (TRK 429; 1.33 TBq/mmol, 36 Ci/mmol) were from Amersham (UK). Sucrose (Aristar grade) was from BDH. All other chemicals were of analytical grade purity and were from Sigma (Switzerland), Calbiochem (USA) or BDH (UK).

Methods

Isolation of plasma membranes from brown adipose tissue (BAT)

Syrian hamsters were cold-acclimated for 4–10 weeks at 4 °C as described previously (Svoboda *et al.* 1993, 1996) and a plasma membrane-enriched fraction was isolated from BAT of control and cold-acclimated hamsters by minor modification of the method described in detail previously (Svoboda *et al.* 1993). Tissue homogenate (1–2 g wet weight of tissue in 10 ml 140 mM NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM MgCl_2 , 1 mM EDTA, 100 $\mu\text{g}/\text{ml}$ phenylmethylsulphonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin) was prepared in a tightly fitting Elvehjem-Potter homogenizer (Teflon-glass, 7 min), filtered through a double layer of silk-cloth, centrifuged for 5 min at 600 g and the resulting supernatant (7 ml) was applied to a discontinuous sucrose density gradient consisting of 43, 35, 31, 27, 23 and 19% w/w sucrose (from bottom to top, 5 ml each) and centrifuged for 60 min at 96 508 g in a Beckman SW28 rotor. The plasma membrane fractions were collected from 23–27% and 27–31% w/w sucrose interphase, diluted 1:1 with 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and the membrane material was sedimented by centrifugation for 60 min at 177 520 g in a Beckman Ti60 rotor. The final membrane pellet was suspended by rehomogenization at a concentration of 5–12 mg membrane protein per ml and stored at –80 °C.

Cell culture and membrane preparations

S49 lymphoma cells (Ransnas *et al.* 1989) and the clones HM1 of CHO (Chinese hamster ovary) cells (Svoboda & Milligan 1994) and E2 of HEK (human embryonic kidney) 293 cells (Kim *et al.* 1994) were grown and plasma membranes prepared as described before.

Production of antisera and immunoblotting

Antisera I325–336, I28–42 and CS3 are rabbit polyclonal antipeptide antisera which were raised in rabbits obtained from VELAZ, Prague, Czech Republic (I325, I28) and in New Zealand White rabbits, Glasgow (CS3) (Mumby *et al.* 1986, Goldsmith *et al.* 1987, Mitchell *et al.* 1989, Milligan 1990). Antisera I325 and I28 were oriented against peptides corresponding to internal sequence of amino acids 325–336 (TPEPGEDPRVTRAKY) or 28–42 (KQLQKDKQVYRATHR) of the $G_s\alpha$ subunit (Mumby *et al.* 1986, Ransnas & Insel 1988, Mumby & Gilman 1991). Antiserum CS3 was raised against a peptide corresponding to the C-terminal decapeptide of $G_s\alpha$, RMHLRQYELL ($G_s\alpha$, 385–394).

Molecular mass determinations were based on prestained molecular mass markers (Sigma, SDS 7B). Standard (10% w/v acrylamide/0.26% w/v bis-acrylamide) or urea (12.5% w/v acrylamide/0.0625% w/v bis-acrylamide containing 6 M urea) SDS-PAGE was carried out overnight at 60 V (10% acrylamide) or 100 V (urea) as described before in detail (Svoboda & Milligan 1994).

Quantification of immunoblots

After SDS-PAGE, proteins were transferred to nitrocellulose and blocked for 4 h in 5% w/v gelatine in PBS, pH 7.4 at 37 °C. Primary antisera were added in 1% w/v gelatine in PBS containing 0.2% w/v Nonidet P40 (NP40) and incubated for at least 4 h at 37 °C. The primary antibody was then removed and the blot washed extensively with PBS containing 0.2% v/v NP40. Secondary antiserum (donkey anti-rabbit immunoglobulin G (IgG) coupled to horseradish peroxidase, Scottish Antibody Production Unit, Wishaw, Scotland, UK) was added (1:200 in 1% gelatine in PBS containing 0.2% NP40) and incubated with nitrocellulose for 2 h. The antiserum was then removed and, after extensive washing of the blot with PBS containing 0.2% NP40 and finally with PBS alone, the blot was developed with *o*-dianidizine hydrochloride (Sigma) as a substrate for horseradish peroxidase as previously described (Mitchell *et al.* 1989). Alternatively, 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 100 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate plus 200 μ g/ml nitro-blue tetrazolium as substrate (Novotný *et al.* 1995).

The developed blots were scanned by BioRad GS 670 imaging densitometer or UMAX Astra 610P to enable quantification of the immunoblots by the ImageQuant 3.3. program.

[3H]CGP12177 binding

Membrane aliquots (0.3–0.8 mg per ml) were incubated for 60 min at 37 °C with [3H]CGP12177 with or without competing ligand (100 μ M L-propranolol) in a total volume of 0.4 ml 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM ascorbic acid. After dilution with ice-cold 50 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$, samples were immediately filtered through fibreglass filters (Whatman GF/C) presoaked in 0.2% polyethylenimine using a Brandell M 24R harvester as described before by Muzzin *et al.* (1992). Saturation binding experiments were performed with varying concentrations of radioligand; in the one-point assay, a total concentration of 30 nM [3H]CGP12177 was used. Non-specific binding was defined as that occurring in the presence of 100 μ M L-propranolol and represented 50–70% of the total binding. All assays were performed in triplicate.

[3H]Ouabain binding

The specific [3H]ouabain binding was measured as described before in detail (Svoboda *et al.* 1988, 1993). Brain microsomes (50–100 μ g per assay) or BAT plasma membrane preparations (100–200 μ g per assay) were incubated with increasing concentrations of [3H]ouabain in a total volume of 0.4 ml 5 mM NaH_2PO_4 , 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.4 ($Mg+P_i$ medium) for 90 min at 37 °C. The binding reaction was discontinued by dilution with 5 ml ice-cold $Mg+P_i$ medium and immediate filtration through Whatman GF/C filters. The filters were dried overnight at laboratory temperature and radioactivity determined by liquid scintillation. The samples of BAT from control and cold-acclimated hamsters were also compared by a one-point assay using a total concentration of 30 nM [3H]ouabain. Non-specific binding was determined in the presence of 10 μ M unlabelled ouabain and accounted for less than 10% of the total binding.

Na,K-ATPase and succinate dehydrogenase enzyme assays

BAT plasma membrane fractions were preincubated for 10 min at 0–4 °C with 0.01% w/v cholate (5 mg protein/ml), 20 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The cholate-treated membranes were diluted into ATPase reaction media A (100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.4) and B (120 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.4, 2×10^{-4} M ouabain) and incubated for 7 min at 37 °C. The enzyme reaction was then started by the addition of ATP (2.5 mM final concentration) and continued for 30 min. Sodium plus potassium activated, ouabain dependent adenosine triphosphatase (EC 3.6.1.3) was determined as positive difference of inorganic phosphate production between Na+K+Mg (A) and Na+Mg+ouabain (B) media as described before in detail (Svoboda & Mosinger 1981).

Succinate dehydrogenase was measured as succinate-cytochrome c oxidoreductase according to the method of King (1967).

Data analysis

Saturation binding experiments and statistical analysis of radioligand binding data were analysed with GraphPad InPlot 4.0. Unless otherwise specified, data are presented in figures and tables as means $\pm$ S.E. of at least three independent experiments performed in triplicate.

RESULTS

The short and long variants of G_{α} proteins

Plasma membrane-enriched fractions were prepared from control and cold-acclimated hamsters, subjected to SDS-PAGE on standard (10% acrylamide/0.26% bis-acrylamide) gels and G_{α} proteins were detected by immunoblotting with I325–336 antiserum. As demonstrated in Fig. 1, the immunoblot signals corresponding to both the long ($G_{\alpha}L$, 52 kDa) and short ($G_{\alpha}S$, 45 kDa) variants of G_{α} were clearly detected. Their identification was carried out by comparison with G_{α} proteins present in membranes isolated from S49 lymphoma cells. Immunoblotting with I28–42 antiserum produced similar results (data not shown).

$G_{\alpha}L$ and $G_{\alpha}S$ proteins were also resolved and identified by urea SDS-PAGE (12.8% acrylamide/0.06% bis-acrylamide/6 M urea) (Fig. 2). Under these conditions, HEK 293 (clone E2) and CHO cells (clone HM1) were used as positive standards. In E2-HEK 293 cells, $G_{\alpha}S$ represents the major form of G_{α} proteins (see note # in legend to

FIGURE 1. Identification of the long ($G_s\alpha L$) and short ($G_s\alpha S$) variants of $G_s\alpha$ protein by immunoblotting with I325–336 antiserum (oriented against an internal sequence of amino acids 325–336). Plasma membranes (6 controls and 6 from cold-acclimated animals) were isolated in parallel from mixed samples of BAT and resolved by standard SDS-PAGE. $G_s\alpha$ proteins were detected by immunoblotting with I325–336 antiserum and alkaline phosphatase based detection of immunoblot signal (see Methods); 100 μ g protein were applied to each lane. S49, immunoblot signal of plasma membrane derived from S49 lymphoma cells (50 μ g protein); W, controls; C, cold-acclimated.

FIGURE 2. Identification of the long ($G_s\alpha L$) and short ($G_s\alpha S$) variants of $G_s\alpha$ protein by immunoblotting with CS3 antiserum (oriented against the C-terminal decapeptide of $G_s\alpha$). Plasma membrane preparations were isolated as described in the legend to Fig. 1 and resolved by urea SDS-PAGE. $G_s\alpha$ proteins were detected by immunoblotting with CS3 antiserum and peroxidase based detection of immunoblot signal (see Methods); 150 μ g protein were applied to each lane. E2, immunoblot signals of $G_s\alpha L$ and $G_s\alpha S$ in clone E2 of HEK 293 cells ($^{\#}$ both signals exhibit slightly lower mobility because this sample was applied in the left corner of the gel); HM1, the clone HM1 of CHO cells; W, membranes from control animals; C, membranes from cold-acclimated animals.

Fig. 2), while in HM1-CHO cells the long isoform $G_s\alpha L$ is dominant (see Methods, Mullaney *et al.* 1993a,b, Kim *et al.* 1994).

In control samples (Fig. 1 and Table 1A), the immunoblot signal of $G_s\alpha L$ was approximately two times higher than that of $G_s\alpha S$ and cold acclimation was reflected by a selective decrease in the short variant, $G_s\alpha S$. The long variant $G_s\alpha L$ was not

changed (Table 1A). Due to this disproportionate change, the $G_s\alpha S/G_s\alpha L$ ratio decreased from 0.48 ± 0.04 in controls to 0.24 ± 0.03 ($P < 0.01$) in cold-acclimated samples. The total amount of both proteins ($G_s\alpha L + G_s\alpha S$) decreased to 83% ($P < 0.05$).

The data obtained by electrophoretic resolution by standard SDS-PAGE and an antiserum oriented

TABLE 1. Effect of cold-acclimation on BAT plasma membrane levels of $G_s\alpha$ proteins. The data represent the average of 12 (6 controls and 6 cold-acclimated) independent plasma membrane preparations $\pm$ S.E. resolved by standard (A) or urea (B) SDS-PAGE. The numbers represent the densitometric scans (expressed in arbitrary units) of immunoblots obtained after resolution and electrophoretic transfer of 100 μ g (A) or 150 μ g (B) membrane protein

	$G_s\alpha$ L	$G_s\alpha$ S	$G_s\alpha$ L + $G_s\alpha$ S	$G_s\alpha$ S/ $G_s\alpha$ L
A Standard SDS-PAGE and I325 antiserum				
Control	34.3 $\pm$ 0.7 (100%)	16.5 $\pm$ 0.9 (100%)	50.8 $\pm$ 1.6 (100%)	0.48 $\pm$ 0.04
Cold	33.8 $\pm$ 1.0 (99%)	8.3 $\pm$ 0.7 (50%)*	42.1 $\pm$ 1.7 (83%)*	0.24 $\pm$ 0.03**
Cold/control	1.0 $\pm$ 0.1	0.5 $\pm$ 0.1**	0.8 $\pm$ 0.1	
B Urea-SDS-PAGE and CS3 antiserum				
Control	16.9 $\pm$ 0.6 (100%)	7.1 $\pm$ 0.9 (100%)	24.0 $\pm$ 1.5 (100%)	0.41 $\pm$ 0.04
Cold	12.5 $\pm$ 0.7 (74%)*	4.0 $\pm$ 0.5 (56%)*	16.4 $\pm$ 1.2 (68%)*	0.31 $\pm$ 0.03*
Cold/control	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1*	0.7 $\pm$ 0.1	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; significant difference between control and cold-acclimated samples (Student's t -test).

against an internal sequence of $G_s\alpha$ were verified by urea SDS-PAGE (Table 1B) and an antiserum oriented against the C-terminus of this protein. Under these conditions, the $G_s\alpha$ S/ $G_s\alpha$ L ratio decreased from 0.41 ± 0.04 to 0.31 ± 0.03 ($P < 0.05$) and the total amount of $G_s\alpha$ proteins was decreased to 68% ($P < 0.01$). The difference between the results obtained by standard SDS-PAGE and urea SDS-PAGE (see Table 1) was apparently related to the effect of concentrated urea on the conformation of $G_s\alpha$ subunits and/or the different sensitivity of antibodies.

Thus, as evidenced by both standard SDS-PAGE and urea SDS-PAGE (Figs 1, 2 and Table 1), cold acclimation of hamster BAT is associated with decrease in plasma membrane density of the short variant of $G_s\alpha$ protein, when compared with the long variant of this protein.

Cold-induced change in composition of plasma membranes

In the next step of our experimentation, the content of other plasma membrane constituents, specific [3 H]CGP12177 binding sites (as an estimate of an overall population of the beta-adrenergic receptors), [3 H]ouabain binding and Na,K-ATPase activity were compared in plasma membrane samples from control and cold-acclimated animals. We have also measured mitochondrial contamination in these membranes.

[3 H]CGP12177 binding

To get the primary information about the binding characteristics of membranes involved in this study, the [3 H]CGP12177 binding experiments were carried out first over a wide range of radioligand concentrations (Fig. 3A). Membranes prepared from cold-acclimated animals exhibited 1.5–2 times

higher specific binding in the whole range of [3 H]CGP12177 concentrations (0.1–87.2 nM). A similar cold versus control difference was obtained from saturation binding experiments carried out with another two plasma membrane preparations, but the absolute values of [3 H]CGP12177 binding were different among these membranes (data not shown).

This surprising result, when related to our previously published data (Svartengren *et al.* 1984, Svoboda *et al.* 1984b), was verified in a highly representative set of 32 independent gradient purified plasma membrane preparations (16 from controls and 16 from cold-acclimated animals). To compare the specific [3 H]CGP12177 binding in all these membranes, a single concentration (30 nM) of this radioligand was used. The results presented in Fig. 3B clearly show a highly significant increase in [3 H]CGP12177 binding in the cold-acclimated group, i.e. from 25 ± 2 fmol/mg in controls to 37 ± 3.0 fmol/mg in cold-acclimated samples ($P < 0.01$). Taken together, these results indicate that the specific [3 H]CGP12177 binding sites present in plasma membrane fractions used in this study were higher in preparations from cold-acclimated animals than in controls.

Plasma membrane markers ([3 H]ouabain binding and Na,K-ATPase activity)

[3 H]Ouabain binding represents a convenient plasma membrane marker in those tissues where Na,K-ATPase, an integral plasma membrane protein and receptor for cardiac glycosides, is present in reasonable quantity and affinity. For instance, [3 H]ouabain binding to brain microsomes proceeds according to the best principles of textbook pharmacology, exhibiting almost no non-specific binding, clear-cut saturation and a large proportion of bound-to-free radioactivity (Svoboda *et al.* 1988,

FIGURE 3. [^3H]CGP12177 binding to BAT plasma membranes isolated from control and cold-acclimated hamsters. (A) Saturation binding curve. Membranes (0.8 mg protein per sample) from control (○) or cold-acclimated (●) animals were incubated with increasing concentrations of radioligand (0.1–87.2 nM; free) according to Muzzin *et al.* (1992). The bound and free radioactivity was separated by rapid filtration through Whatman GF/C filters and specific and non-specific [^3H]CGP12177 binding determined as described in Methods. The data represent the single binding curve determined in triplicate. (B) One-point assay in membranes prepared from control (W) and cold-acclimated (C) animals. The [^3H]CGP12177 binding was determined by one-point assay using 30 nM (total) concentration of radioligand. The numbers represent the average $\pm$ S.E. of 32 gradient purified plasma membranes of BAT (16 controls and 16 cold-acclimated).

FIGURE 4. [^3H]Ouabain binding to BAT plasma membranes. Membranes (200 μg protein per assay) from control (○) or cold-acclimated (●) animals were incubated with increasing concentrations of [^3H]ouabain and the bound and free radioactivity were determined as described in Methods. (A) Saturation binding curve. (B) Scatchard plot analysis of the binding data. The data represent the single binding curve determined in duplicate.

1993). Therefore, the number of [^3H]ouabain binding sites may be used as an internal standard of plasma membrane content in a given subcellular membrane fraction.

The situation in brown fat is less favourable than in brain, but the saturation binding experiments may be performed easily (Fig. 4) and the number of sites compared in various membrane preparations (Fig. 5). This approach was used to compare plasma membrane preparations isolated from control and cold-acclimated animals.

Specific [^3H]ouabain binding was increased roughly 3 times in plasma membranes prepared from cold-acclimated hamsters and this increase was

FIGURE 5. Comparison of [^3H]ouabain binding (one-point assay) and Na,K-ATPase activity in various plasma membrane preparations of BAT. Plasma membranes (4 controls (W) and 4 from cold-acclimated animals (C)) were isolated in parallel from mixed samples of BAT and (A) the [^3H]ouabain binding (fmol/mg protein) and (B) the Na,K-ATPase enzyme activity ($\mu\text{mol P}_i \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$) were measured as described in Methods. The data represent the average $\pm$ s.e. of the radioligand binding or enzyme activity assays carried out in triplicate.

observed in the whole range of [^3H]ouabain concentrations, 1–211 nM (Fig. 4). Comparison of the four plasma membrane preparations (measured by one-point assay at 30 nM [^3H]ouabain) indicated a significant increase, from 188 ± 19 to

398 ± 48 fmol/mg (Fig. 5A). Very similar data were obtained for Na,K-ATPase activity, which was increased from 1.38 ± 0.14 to 2.64 ± 0.28 $\mu\text{mol P}_i \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ (Fig. 5B).

Mitochondrial markers

To ensure that the mitochondrial biogenesis known to occur during cold acclimation did not lead to an increased mitochondrial contamination of plasma membrane fractions involved in this study (which could partly explain the reductions in G protein levels described above), the level of mitochondrial contamination was followed by measurements of succinate-cytochrome c reductase (see Methods). Whereas – as expected – the mitochondrial activity in crude membrane preparations from cold-acclimated animals was higher (≈ 2.5 times) than in crude membranes from controls (Table 2), the mitochondrial activity in the purified plasma membrane fractions was identical in the two cases (≈ 29 nmol cytochrome c per min per mg protein) (Table 2). Thus, increased mitochondrial contamination could not explain the reduction in G protein levels reported here.

DISCUSSION

Purification of plasma membranes from BAT

Plasma membranes isolated previously on continuous sucrose density gradients (Svoboda *et al.* 1984b) from cold-acclimated hamsters exhibited much lower activity of the plasma membrane markers Na,K-ATPase (26%) and 5'-nucleotidase (36%) than membranes isolated from control animals. Simultaneously, these membranes contained higher activity of mitochondrial enzymes, succinate-cytochrome c reductase (1.4 times) and α -glycerolphosphate dehydrogenase (1.5 times). Under these conditions, the number of specific [^3H]dihydroalprenolol binding sites was decreased by 34%. Similar data were obtained on 3 times washed crude membranes (sediment 100 000 g) (Svartengren *et al.* 1984).

Throughout the years, we have tried to improve this isolation procedure. Based on experience obtained from subcellular fractionation of S49 lymphoma cells (Svoboda *et al.* 1992), the new method for isolation of plasma membrane from BAT was introduced (Svoboda *et al.* 1993). This method used non-equilibrium centrifugation on discontinuous gradients composed of sucrose solutions of exactly defined volumes and densities. The purity of plasma membrane-enriched fractions was, under these conditions, substantially

TABLE 2. Succinate-cytochrome c reductase (expressed as nmol cytochrome c reduced/min/mg protein) in membrane fractions of BAT

	Crude membranes		Gradient purified plasma membranes	
	Control	Cold	Control	Cold
Preparation				
I	16.7	25.0	24.1	23.4
II	10.2	23.2	34.1	34.7
III	10.9	27.0	16.1	16.2
IV	8.5	19.2	39.8	41.9
Average	11.6 ± 1.5	23.6 ± 1.4**	28.5 ± 4.6	29.1 ± 5.0 NS

Crude membranes: The BAT homogenate was filtered through the double layer of silk-cloth, centrifuged for 5 min at 1500 r.p.m. and the resulting supernatant was centrifuged for 60 min at 50 000 r.p.m. in a Beckman Ti50 rotor. Resulting sediment was rehomogenised in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA at 15–20 mg protein/ml. Gradient purified plasma membranes (four independent preparations, I–IV) were prepared as described in Methods. The difference between control and cold-acclimated samples was analysed by Student's *t*-test; ** indicates significant effect of cold-acclimation (*P*<0.01); NS, not significant.

improved. The mitochondrial contamination was roughly the same and isoprenaline-stimulated adenylyl cyclase as well as G_sα functional activity (cyc[−] reconstitution assay) was decreased in cold-acclimated samples. Surprisingly, the number of [³H]ouabain binding sites was increased (Svoboda *et al.* 1993). This new method was used in the present work for further analysis of the effect of cold-acclimation on composition of BAT plasma membranes. Both Na,K-ATPase activity and [³H]ouabain binding sites were measured and found to be increased in parallel. Furthermore, the number of specific [³H]CGP12177 binding sites in these improved membranes was also increased.

Therefore, it may be concluded that, from a structural point of view, cold-acclimation of BAT (long-term adaptation to cold) induces a disproportionate change in plasma membrane components – the membrane density of G_sα proteins (immunoblot analysis) is decreased while [³H]ouabain binding, [³H]CGP12177 binding and Na,K-ATPase activity are increased. The decrease in G_sα proteins proceeds preferentially as a decrease in the short variant of G_sα. From the functional point of view, the cold-induced desensitisation of BAT (Freidli *et al.* 1978, Nedergaard 1982, Svartengren *et al.* 1982, Svartengren *et al.* 1984, Unnelius 1990, 1993, Kuroshima *et al.* 1991) is associated with decreased beta-adrenergic responsiveness of adenylyl cyclase and by decreased functional activity of G_sα proteins (Svoboda *et al.* 1993).

The (non)identity of the short and long variants of G_sα

In the past decade a lot of controversial data have been gathered about function, distribution or

behaviour of the short and long variants of G_sα (for review see Novotný & Svoboda 1998). The notion about unequal functional properties and/or behaviour of the two G_sα variants still remains a matter of debate and awaits further experimental support. Dramatic changes in the levels of G_sαS and G_sαL have been reported under various physiological (e.g. development, ageing and cellular differentiation) and pathophysiological (e.g. hepatectomy, alcoholism and genetic disorders) conditions (Palmer & Houslay 1991, Rius *et al.* 1991, Urasawa *et al.* 1991, McFarlane-Anderson *et al.* 1992, Ozawa *et al.* 1993, Michel *et al.* 1994, Viollet *et al.* 1994, Yagami *et al.* 1994, Denis-Henriot *et al.* 1996, Kawai & Arinze 1996). This evidence, although descriptive and indirect, certainly promotes the idea that the expression of G_sα splice variants is regulated according to the functional needs of a given cell or tissue.

The non-identical behaviour (distribution) of the short and long G_sα variants has also been described in adipose tissue. Quantification of G_sα proteins in white adipose tissue of genetically diabetic (db/db) mice revealed that G_sαL was significantly less abundant than in non-diabetic controls, whereas there was no difference in G_sαS (Begin-Heick 1992). A similar result was obtained for genetically obese (ob/ob) mice compared with lean littermates (Begin-Heick 1990). Preferential change of the short isoform was found in white adipocytes of aged rats, which exhibited a marked increase in G_sαS detected by both immunoblotting and cholera toxin-induced ADP ribosylation (Green & Johnson 1989). Interestingly, an identical reduction in both G_sα subforms was also reported in the adipocytes of obese (fa/fa) Zucker rats (Strassheim *et al.* 1991).

Another line of evidence in favour of differential regulation of the steady-state levels of G_sα variants, which is more directly related to the problem of our current study, has been described by Granneman *et al.* (1990). The perinatal stimulation (recruitment) of BAT was associated with changes in the splicing pattern of G_sα mRNA, and these changes were reflected in G_sα protein expression. While G_sαL mRNA increased significantly and G_sαL protein levels remained unchanged, G_sαS mRNA did not change and G_sαS protein declined to 40% of the control level. The decrease in the G_sαS/G_sαL ratio was therefore achieved by preferential decrease in G_sαS, with little or no change in G_sαL (Chaudhry & Granneman 1991).T

Thus, both types of recruitment of BAT, i.e. cold acclimation and perinatal cold stress, are accompanied by a similar change in the relative proportion between the short and long variants of G_sα. Preferential change in G_sαS supports the idea that the steady-state level of this G_sα variant is more readily or actively regulated by recruitment of BAT.

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