Cold-induced reduction in $G_i\alpha$ proteins in brown adipose tissue

Effects on the cellular hypersensitization to noradrenaline caused by pertussis-toxin treatment

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The significance of G_i proteins for the physiological desensitization phenomena observed in brown-fat cells from cold-acclimated hamsters was investigated. For this purpose, pertussis toxin (the inhibitor of G_i function) was injected into control and cold-acclimated hamsters. After 3 days the thermogenic response to noradrenaline injection was monitored in the intact animals. It was found that the pertussis-toxin pretreatment did not affect the thermogenic response to noradrenaline. Nonetheless, the pertussis toxin pretreatment had a dramatic effect on the noradrenaline-sensitivity of isolated brown-fat cells (measured the following day as the respiratory response): a 250-fold-increased sensitivity to noradrenaline was observed in cells from control animals that had been pertussis-toxin pretreated. However, only a 20-fold increase was observed in cells from cold-acclimated hamsters, implying a lower complement of the G_i system in these cells. Therefore the content of G_i proteins was determined by

quantitative immunoblotting of purified plasma-membrane proteins. Cold acclimation resulted in a nearly 50 % reduction in the content of $G_{i1}\alpha$ and $G_{i2}\alpha$, as well as of the β -subunit, both when expressed on a protein basis and when related to the content of forskolin-stimulated adenylyl cyclase; when expressed per unit of [3 H]ouabain-binding (Na^+/K^+ -ATPase), the reduction was even higher. In view of the magnitude of the pertussis-toxin effect, it was concluded that G_i proteins must play a substantial role in the regulation of the response of brown-fat cells to noradrenaline. As the capacity of the G_i pathway is reduced rather than augmented during cold acclimation, G_i activity cannot be responsible for the desensitization to noradrenaline observed in cells from cold-acclimated animals. However, the reduced G_i content may explain the earlier observed desensitization to adenosine that occurs after acclimation to cold.

INTRODUCTION

The phenomenon of desensitization has been described for a variety of hormone receptors, but the phenomenon has especially been studied in the context of G-protein-coupled receptors, notably adrenergic receptors [1–5]. In general, studies of desensitization have been performed *in vitro*, and few studies have attempted to extend the understanding obtained from the *in vitro* studies to *in vivo* conditions.

Brown adipose tissue provides an interesting opportunity to study the molecular mechanism of desensitization to adrenergic stimuli under *in vivo* conditions. Brown adipose tissue produces heat when it is stimulated by noradrenaline released from the sympathetic nervous system [6,7] and it is thus constantly adrenergically activated when an animal is exposed to cold.

That desensitization is a phenomenon that does occur as an effect of adrenergic stimulation *in vivo* can be deduced from the fact that cells isolated from brown adipose tissue of cold-acclimated animals have an ≈ 10 -fold lower sensitivity to noradrenaline than cells from control animals (i.e. a 10-fold higher EC_{50}) [8–11]. We earlier investigated different aspects of this physiologically induced functional desensitization. We have, for example, presented evidence that the functional activity and content of G_s -protein is decreased [12], and that the phosphodiesterase activity is enhanced [11].

A further possibility would be that the inhibitory influence elicited via G_i -proteins on adenylyl cyclase activity would be increased by cold acclimation. We have therefore here tested whether such an increase in the capacity of the inhibitory pathway is induced by cold acclimation. We have examined the occurrence

of such effects in two ways: we have attempted to inhibit the G_i -mediated inhibitory pathways with pertussis toxin, and we have examined the content of $G_i\alpha$ proteins in the tissue with specific antibodies.

MATERIALS AND METHODS

Pertussis-toxin pretreatment

Adult (routinely 23–28 weeks old) Syrian hamsters, equal numbers of both sexes, from the Wenner-Gren Institute strain were singly caged, for at least 4 weeks either at 4 °C (cold-acclimated) or for the same time at 24 °C (controls). The hamsters had free access to the standard hamster diet of the animal house (based on rabbit/guinea-pig pellets, with added sunflower seeds, dried carrots, wheat-germ and oats) and to water, and they were exposed to a 6 h-light/18 h-dark photoperiod (lights on at 08:00 h) (i.e. conditions similar to those used previously [8–12,14]). After being acclimated, the hamsters were injected intraperitoneally with pertussis toxin (Sigma; 1 μ g/100 g body weight) or with saline (identical volume). Injections and subsequent analyses were always performed in parallel (one saline-plus one pertussis-toxin-treated hamster).

At 3 days after the pertussis-toxin treatment, the hamsters were placed in a metabolic chamber at 24 °C. After ≈ 2 h of habituation to the chamber, the hamsters were injected with noradrenaline, principally as previously described [13,14].

The next (fourth) day, the hamsters were killed, and brown-fat cells were prepared by collagenase digestion and their response to noradrenaline investigated in an oxygen electrode chamber as described previously [11].

Abbreviation used: NP40, Nonidet P40.

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Isolation of plasma membranes from brown adipose tissue

The plasma membranes investigated were the membrane preparations earlier characterized in detail [12]. Briefly, tissue homogenates were prepared in parallel from brown adipose tissue of control and cold-acclimated hamsters (more than 4 weeks at 20 °C and 4 °C respectively). The homogenates were applied on discontinuous sucrose density gradients and centrifuged for 60 min at 85 000 *g*. Gradient fractions were collected, diluted 1:1 with hypo-osmotic Tris buffer, and the membrane material of each fraction was pelleted by centrifugation for 60 min at 150 000 *g*. The plasma-membrane fractions used in the present study were collected from the 23–27% and 27–31% (w/w) sucrose interphases (the combined fractions 3 and 4); these fractions had the highest relative content of plasma membrane, as verified by [³H]ouabain binding and adenyl cyclase activity [12]. The plasma-membrane fractions were stored at a concentration of 5–12 mg of membrane protein/ml at –80 °C and used for analysis of G-protein content as described below. All preparations were paired parallel preparations (one from control and one from cold-acclimated hamsters).

Rat glioma C6 BU1 cells were grown in tissue culture, and a total membrane pellet was prepared as previously described in detail [15]. A total membrane fraction was also prepared from rat cerebral cortex in a manner similar to that used for glioma cells.

Antisera

The production and characterization of the antisera used in this study have been described in detail elsewhere [16–18]. Each antiserum was raised against a synthetic decapeptide corresponding to a sequence in the antigen of interest and coupled to keyhole-limpet haemocyanin (Calbiochem).

Antiserum SG1 was raised against a synthetic peptide corresponding to the C-terminal ten amino acids of transducin:

KENLKDCGLF ($G_{i\alpha}$ 341–350)

Antiserum I3B was raised against a peptide corresponding to the C-terminal region of $G_{i3\alpha}$:

KNNLKECGLY ($G_{i3\alpha}$ 345–354)

Antiserum I1C was raised against a peptide corresponding to amino acids 159–168 of the $G_{i1\alpha}$ subunit:

LDRIAQPNYI

Antiserum BN2 was raised against a synthetic peptide corresponding to the N-terminal decapeptide of the β_1 subunit [19].

Immunological quantification of G-proteins

The plasma-membrane preparations were diluted to equal protein concentrations (5 mg/ml) and then further diluted with an equal volume of twice-concentrated Laemmli sample buffer and left at room temperature for 1 h. The membrane proteins were resolved by 6 M urea/SDS/12.5% acrylamide/0.06% bisacrylamide (w/v) PAGE in 20 cm × 20 cm slab gels run overnight at 35 mA constant current.

After SDS/PAGE, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and blocked for at least 3 h with 5% (w/v) gelatin in PBS (0.27 mM KCl/0.15 mM KH_2PO_4 /137 mM NaCl/4 mM Na_2HPO_4). The primary antiserum was then removed and the blot washed extensively with PBS containing 0.2% (v/v) Nonidet P40 (NP40) and incubated overnight. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; Scottish Antibody Production Unit, Wishaw, Lanarkshire, Scotland, U.K.) was added (1:200 in 1% gelatin in PBS containing 0.2% NP40) and incubated with the

nitrocellulose membranes for at least 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 [17]. On each blot, the spots were densitometrically scanned, and for each parallel pair of preparations (one control and one cold-acclimated hamster), the value obtained with the preparation from the control animal was set to 100% and the value from the cold-acclimated animal expressed relative to this.

RESULTS AND DISCUSSION

Effect of pertussis-toxin pretreatment on brown-adipose-tissue noradrenaline-sensitivity *in vivo*

It could be suggested that an increased capacity of the inhibitory G_i pathway may participate (together with the decreased capacity of the G_s pathway [9,12] and the stimulated phosphodiesterase activity [11]) in the desensitization to adrenergic stimulation which can be observed in brown-fat cells isolated from cold-acclimated animals. If this were the case, it would be anticipated that treatment with the G_i inhibitor pertussis toxin would eliminate any G_i -related difference between cells from cold-acclimated and control animals. It would further be expected that such an inhibition of G_i protein function would increase the noradrenaline-sensitivity of the brown-fat cells both *in vitro* and *in situ*.

The thermogenic process in brown-fat cells *in situ* can be

Figure 1 Absence of effect of pertussis-toxin pretreatment on the thermogenic response to noradrenaline injection in intact hamsters

Hamsters were left under control conditions (A and B) or acclimated to cold (C and D) as described in the Materials and methods section. At 3 days before the experiments shown here, the hamsters were injected with saline (A and C) or pertussis toxin (B and D). On the day of investigation, the hamsters were transferred to the metabolic chamber. During an ≈ 2 h habituation period (not shown) the resting metabolic rate (RMR) was measured (as defined in the legend to Table 1); the level is indicated on the graphs. After the habituation period, the hamsters were injected, first with 0.1 mg, then with 1 mg of noradrenaline ('norepinephrine', NE) bitartrate/kg body weight, and the oxygen consumption followed. The y-axes show the decrease (in %) in the oxygen concentration of the air withdrawn from the chamber [ΔO_2 (%)]. Short-lived peaks indicate physical activity of the hamsters. In connection with each injection, the surrounding air was analysed by the system, giving rise to the artefacts where $\Delta O_2 = 0\%$. Data obtained from a series of hamsters examined in this way are compiled in Table 1.

Table 1 Effect of pertussis-toxin pretreatment and acclimation to cold on the thermogenic response of hamsters to noradrenaline

The Table is based on experiments such as those depicted in Figure 1. At the time of the experiment, the mean weight of the control, saline-pretreated hamsters was 139 ± 11 g, the control, pertussis-toxin (PT)-pretreated 135 ± 6 g, the cold-acclimated, saline-pretreated 110 ± 5 g, and the cold-acclimated, pertussis-toxin-pretreated 113 ± 11 g. The data are expressed as ml of O $_2$ $\cdot$ min $^{-1}$ $\cdot$ kg $^{-0.75}$. The resting metabolic rate was defined as the minimal level of oxygen consumption measured for at least 5 min during the 2 h habituation period before the first noradrenaline (NA) injection (indicated by the line in Figure 1). The level reached after injection of 0.1 or 1.0 mg of noradrenaline per kg body weight was similarly defined as the maximal level of oxygen consumption maintained for at least 5 min after each noradrenaline injection. The ΔV_{O_2} values given in the Table were defined as the difference between the resting metabolic rate (obtained before the first noradrenaline injection) and the rates after each noradrenaline injection. These definitions are identical with those used elsewhere [14] and were formulated to minimize the effects of alterations in physical activity on the data. The data on resting metabolic rate, and on the increase in metabolic rate caused by 0.1 and 1 mg of noradrenaline, were separately analysed with two-way analysis of variance [five or six replicates (hamsters) in each group] for the effect of cold acclimation and of pertussis-toxin pretreatment. For the resting metabolic rate, a significant effect ($P < 0.01$) of cold acclimation was found, but no effect of pertussis-toxin pretreatment and no interaction. For the increase in metabolic rate ΔV_{O_2} (above resting) caused by 0.1 mg of noradrenaline, no significant effects of treatment and no interaction were observed. For the increase in metabolic rate (above resting) ΔV_{O_2} caused by 1 mg of noradrenaline, there was a significant effect of cold acclimation ($P < 0.01$), but not of pertussis-toxin treatment, and no interaction.

Conditions	Resting metabolic rate	ΔV_{O_2}	
		0.1 mg of NA	1 mg of NA
Control, saline-pretreated	9.2 ± 0.3	7.1 ± 1.5	19.0 ± 2.8
Control, PT-pretreated	11.1 ± 1.0	6.1 ± 1.5	20.3 ± 3.4
Cold-acclimated, saline-pretreated	12.3 ± 1.2	9.5 ± 2.9	28.6 ± 5.0
Cold-acclimated, PT-pretreated	14.1 ± 1.0	11.2 ± 3.2	34.1 ± 3.5

elicited by injection of noradrenaline into intact animals and can be monitored by measuring the oxygen consumption of the intact animals [20]. It would therefore be expected that the brown-fat cells in a pertussis-toxin-treated animal would be more sensitive to injected noradrenaline than the cells in control animals.

To test whether such a sensitization to noradrenaline was induced by pertussis-toxin treatment, we investigated the thermogenic response to noradrenaline of intact hamsters which had been either saline-injected or injected with pertussis toxin 3 days earlier. To be able to observe a pertussis-toxin-induced hypersensitivity, we used two doses of injected noradrenaline (bitartrate): the standard 1 mg/kg body weight, which has been demonstrated to lead to full stimulation of thermogenesis in hamsters [14], and a 10-fold lower dose of noradrenaline.

As exemplified in the recording shown in Figure 1A, in a control, saline-pretreated hamster, the response to injection of 0.1 mg of noradrenaline/kg body weight was small and transient; the response was not different from that seen if only the vehicle (saline) was injected (result not shown). However, a clear increase in metabolic rate was observed after injection of 1 mg of noradrenaline. In Figures 1B–1D the responses of hamsters pretreated in the different ways are exemplified.

In Table 1 we have compiled data from a series of experiments performed as exemplified in Figure 1. The mean resting metabolic rate was somewhat higher in the cold-acclimated than in the control hamsters (in agreement with [14]). There was no statistically significant effect of pertussis-toxin pretreatment on the resting metabolic rate.

Also, as previously demonstrated [14], the maximal increase in oxygen consumption (after 1 mg of noradrenaline/kg body weight) in the cold-acclimated hamsters was significantly higher

than that observed in control hamsters (Table 1, last column). Pertussis-toxin pretreatment again did not influence the response.

Concerning the potentially most interesting results, the influence of treatment on the metabolic response to a submaximal dose of noradrenaline (0.1 mg/kg body weight), no effect of either cold acclimation or of pertussis-toxin pretreatment was observed. Thus pertussis toxin did not induce a higher sensitivity to injected noradrenaline. This means that pertussis toxin was apparently not able to abolish an inhibitory function of G $_i$ proteins in the brown-fat cells *in situ* to an extent that influenced the noradrenaline-sensitivity when determined *in vivo*.

It may be noted that this absence of effect was not due to unsuccessful pertussis-toxin treatment, because the cells from the same animals tested in an *in vitro* situation showed a very marked effect of pertussis-toxin pretreatment (see below).

Effect of pertussis-toxin pretreatment on brown-fat-cell noradrenaline-sensitivity

The day after the investigation described above in the intact hamsters, the hamsters were killed. Brown-fat cells were prepared from these animals and examined in the oxygen electrode chamber for their responsiveness to noradrenaline stimulation *in vitro*. The results are displayed in Figure 2 and Table 2.

The well-known desensitization to noradrenaline caused by acclimation to cold was observed: the EC $_{50}$ was significantly higher in cells from cold-acclimated than from control hamsters. The difference between the two groups was not as great as that found previously [8–11]; instead of the expected nearly 10-fold decrease in sensitivity, only a 2–2.5-fold decrease was seen. From further scrutiny of the data it was clear that the absolute EC $_{50}$ value of the cells from the cold-acclimated hamsters was approximately as expected (≈ 300 nM), whereas the EC $_{50}$ of cells from control hamsters was somewhat higher than expected. As described above, all the hamsters had been injected with noradrenaline the previous day to investigate the noradrenaline-sensitivity of the brown-fat cells *in situ*. It cannot be excluded that this short pre-exposure to noradrenaline had initiated a slight desensitization process, and this may perhaps explain the somewhat reduced difference between the adrenergic sensitivity of the cells from the control and the cold-acclimated hamsters observed here.

Pertussis-toxin-induced hypersensitization to noradrenaline

A very remarkable sensitizing effect of pertussis-toxin pretreatment was observed in the isolated brown-fat cells: in control cells the EC $_{50}$ for noradrenaline was lowered ≈ 250 -fold by pertussis-toxin pretreatment, from ≈ 140 nM down to ≈ 0.5 nM (Table 2) (the exact values depend upon the calculation method used, as detailed in the legends to Table 2 and Figure 2). This decrease was thus very much greater than the decrease caused by removal of the only known effector of the G $_i$ pathway in these cells, namely adenosine; removal of adenosine by adenosine deaminase causes only a ≈ 2 -fold decrease in EC $_{50}$ for noradrenaline [10]. It is very surprising that the large pertussis-toxin-induced hypersensitivity was not reflected in the responsiveness of the intact hamster to a low dose of noradrenaline (Figure 1 and Table 1), and we see no simple explanation for this.

From this effect of pertussis toxin in the isolated cells, it may be implied that the pertussis-toxin substrates, i.e. the G $_i$ proteins, have an important regulatory effect on adrenergic sensitivity in these cells. A tendency to an increased agonist sensitivity has been reported by Schimmel et al. [21] to occur after incubation of

Figure 2 Effect of pertussis-toxin pretreatment on the response of isolated brown-fat cells to stimulation with noradrenaline

Cells were isolated from the hamsters which had been left under control conditions (**A**) or acclimated to cold (**B**) as described in the Materials and methods section and whose metabolic response to noradrenaline had been investigated the previous day (Figure 1). At 4 days before cell isolation, the hamsters had thus been injected with pertussis toxin (●, ■) or with saline (○, □). The cells were isolated as described in the Materials and methods section and investigated in the oxygen electrode chamber. Noradrenaline was successively added to the indicated concentrations. Points are means $\pm$ S.E.M. for three to five cell preparations in each condition. (*), * and ** indicate significant effects of pertussis-toxin pretreatment ($P < 0.1$, 0.05 and 0.01 respectively; Student's unpaired t test). The curves were calculated by the reiterative general curve-fitting program of the Kaleidagraph data analysis/graphics application for Macintosh for best fit to the Michaelis-Menten-type equation:

$$V_x = V_{\text{basal}} + V_{\text{max}} \cdot [x / (x + EC_{50})]$$

In (**A**), the EC_{50} values obtained in this way (i.e. from the curve drawn, based on the mean values) were 81 ± 15 nM for cells from control hamsters pretreated with saline and 0.16 ± 0.06 nM for cells from hamsters pretreated with pertussis toxin (i.e. a 506-fold improved noradrenaline-sensitivity); in (**B**), the EC_{50} values were 209 ± 91 nM for cells from cold-acclimated hamsters pretreated with saline and 9 ± 4 nM for those pretreated with pertussis toxin (i.e. a 23-fold improved noradrenaline-sensitivity). See also Table 2 for the results of an alternative calculation method.

isolated brown-fat cells with pertussis toxin *in vitro*, but the effect caused by such *in vitro* treatment was low compared with the large sensitization observed here with pertussis-toxin pretreatment *in vivo*. However, Wesslau and Smith [22] have observed a similar large sensitization of the lipolytic response of white adipocytes as a result of pertussis-toxin pretreatment of rats (whereas the sensitizing effect of *in vitro* pertussis-toxin treatment of white adipocytes is also minor [23]); thus it would seem that much more marked effects are seen of *in vivo* pertussis-toxin pretreatment than of *in vitro* treatment.

Decreased pertussis-toxin-induced hypersensitization due to cold acclimation

In brown-fat cells isolated from cold-acclimated hamsters also, a sensitizing effect of pertussis toxin was seen, from an EC_{50} for noradrenaline of ≈ 300 nM down to ≈ 15 nM. It is, however, evident that the effect was smaller than that seen in control cells (Figure 2). Thus, as compiled in Table 2, the decrease in EC_{50} caused by pertussis toxin was significantly reduced: in the cells from cold-acclimated hamsters it was one order of magnitude smaller than in cells from control animals (≈ 20 -fold versus ≈ 250 -fold).

The observation of a decreased effect of pertussis-toxin pretreatment on the sensitivity of cells from cold-acclimated hamsters to noradrenaline is contrary to expectations if the desensitization in cells from cold-acclimated animals is due to an increased capacity of the inhibitory receptor/adenylyl-cyclase pathway. Pertussis-toxin pretreatment was thus not able to reduce the difference in noradrenaline-sensitivity between the two types of cells. Rather, the pertussis-toxin pretreatment augmented the difference (by about 10-fold) between the two cell types, in this respect (Table 2). Thus we see no evidence for an increased capacity of the inhibitory receptor/adenylyl cyclase pathway as an explanation for the desensitization to noradrenaline stimulation observed in isolated brown-fat cells from cold-acclimated animals.

The identification of G_i proteins in brown adipose tissue

A molecular explanation for the decreased effect of pertussis-toxin pretreatment in the cold-acclimated hamsters could be that a decrease in the content of the pertussis-toxin substrate, i.e. the G_i proteins, had occurred during cold acclimation. We therefore characterized and measured the G_i proteins present in the brown adipose tissue of control and cold-acclimated animals.

There is good evidence in the literature for the presence of functional amounts of G_i protein in brown adipose tissue. Thus inhibitory effects of α_2 -adrenergic stimulation, expected to be mediated via the G_i pathway, have been observed in isolated rat brown-fat cells [24] (although not in hamster brown adipocytes such as those used in the present study [25]) and in cultured rat brown-fat cells [26]. Similarly, adenosine, probably via A_1 receptors and G_i proteins, has been shown to inhibit noradrenaline-stimulated oxygen consumption, both in rat brown-fat cells [27–29] and in hamster brown-fat cells [10,30,31]. Further, the effects of pertussis toxin in itself, as reported above, and by Schimmel [21], are indicative of functional influences of G_i -dependent pathways.

The presence of G_i proteins as such has been indicated through pertussis-toxin-induced ribosylation of membrane proteins. Schimmel et al. [21] observed pertussis-toxin-mediated [32 P]ADP-ribosylation of a protein with a molecular mass of ≈ 42 kDa in membranes from brown-fat cells, and Granneman [32] observed ribosylation of an ≈ 39 kDa protein but noted that 'cold exposure did not influence the ability of pertussis toxin to label this protein'. In a more detailed analysis, Granneman and colleagues [33] found pertussis-toxin-mediated ribosylation of 40 and 41 kDa proteins, the 40 kDa protein being more labelled than the 41 kDa, and a postnatal decrease in the level of ribosylation of both bands. Also, the presence of mRNA corresponding to $G_{i1\alpha}$ and $G_{i2\alpha}$ has been observed [34].

For the present study, we chose to use previously characterized specific antibodies to investigate and quantify G_i proteins in brown adipose tissue. We primarily used the antiserum SG1 raised against the C-terminal decapeptide of transducin. Trans-

Table 2 Effect of pertussis-toxin pretreatment and acclimation to cold on the noradrenaline sensitivity of isolated brown-fat cells

The Table is based on the same experiments as those depicted in Figure 2. However, here, for each individual cell preparation, the dose-response relationship was analysed with the equation described in the legend to Figure 2 and the EC₅₀ value for this particular preparation thus calculated. The mean EC₅₀ values presented here were then calculated from the pEC₅₀, leading to asymmetric SE when recalculated to arithmetic EC₅₀ values; therefore the mean $\pm$ S.E.M. is given here as a range (in parentheses). The values are from four or five cell preparations in each group. (A) These ratios were calculated from the tabulated mean values. (B) These ratios were calculated such that the relative effect of pertussis-toxin pretreatment [i.e. the ratio EC₅₀(saline)/EC₅₀(pertussis toxin)] was calculated for each experimental day, and the mean and S.E.M. of these (logarithmized) values were then calculated. † and †† indicate significant effects of cold acclimation ($P < 0.05$ and 0.01 respectively; Student's unpaired t test on the pEC₅₀ values), and ** and *** indicate significant effects of pertussis-toxin pretreatment ($P < 0.01$ and 0.001 respectively; Student's unpaired t test on the pEC₅₀ values).

	Saline	Injection ...	Pertussis toxin	(A) Saline*	(B) Saline*
				pertussis toxin	pertussis toxin
Control	143 (125–162)		0.5 (0.2–1.4)***	246	200 (91–438)
Cold-acclimated	301 (255–356)††		17 (7–43)**	18	11 (5–25)†
Cold-acclimated/control	2.1		34	0.07	0.06

ducin is not present in brown adipose tissue, but antiserum to the transducin decapeptide has earlier been successfully used to estimate the content of G α proteins in different tissues [16,35,36], including white adipose tissue [17,19,37]. This is feasible because both G α 1 and G α 2 differ from the transducin sequence by only one amino acid (a glutamic acid-to-asparagine substitution in the ninth residue). G α 3 has three substitutions in this sequence and is not recognized by this antiserum [38], and G α proteins have even less similarity.

Figure 3A shows immunoblots with the SG1 antiserum of purified plasma membranes isolated from brown adipose tissue of control (W) and cold-acclimated (C) hamsters. As seen, the SG1 antiserum reacted with two proteins in the brown-fat plasma membranes, with molecular masses of approx. 41 and 40 kDa. The band with greater mobility through the gel co-migrated with the major G α type protein of rat glioma C6 BU1 cells, which has previously been shown to correspond to G α 2 [17], whilst the polypeptide with lower mobility co-migrated with the major form of G α from cerebral cortex, G α 1 [17]. Therefore the two bands were identified as the G α 1 and G α 2 subunits respectively.

A third band, termed 'NSP' in Figure 3A, was also recognized by the SG1 antiserum in plasma membranes of brown adipose tissue, but this band had no counterpart in brain homogenate. The nature of this band is unknown. It would appear that the immunological interaction as such is specific, that the peptide has a molecular mass (34–35 kDa) only slightly lower than the other G α -proteins and that a weak but distinct counterpart was visible in C6 cells. It should be remembered that the brown-fat fractions are purified plasma-membrane fractions, thus excluding that this is a non-plasma-membrane peptide. Further, this peptide was more abundant than G α 1 in brown adipose tissue and (as demonstrated below) appeared to show differential expression due to physiological stimulus. In the Swiss Protein Sequence Bank there is no entry of any characterized protein with a closer sequence similarity to the decapeptide sequence used for the antibody production than G α or G α 3 (which, as stated above, are not recognized by this antibody). Thus the NSP band may perhaps represent a post-translational modification of G α 1 or G α 2 or a so-far-uncharacterized plasma-membrane protein. The possibility that the band represents a proteolytic degradation product cannot fully be excluded, but the experimental conditions utilized make this possibility less likely.

We also made immunoblots with the antiserum IIC, which is G α 1-specific [17]. However, with this antiserum, no strong positive immunoreaction was observed (principally in agreement

with the low amount of G α 1 detected with the SG1 antiserum used above) (result not shown).

In an attempt to observe the presence of the third G α type, G α 3, we used the antiserum I3B. With this antiserum we were unable to detect a positive immunoreaction with the brown-fat plasma-membrane fractions. Therefore the existence of G α 3 in brown adipose tissue could be neither proven nor disproven. G α 3 has earlier been identified in white adipocytes with this antibody [17,19,37] and at the mRNA level [39–41], but there are several indications that G α 3 may not be present in brown adipose tissue [33,34].

As regards the relative content of G α proteins, it is clear from the immunoblot (Figure 3A) that, in comparison with rat brain, brown adipose tissue contains relatively low amounts of both G α 1 and G α 2. Despite the fact that a 4-fold higher amount of protein was loaded in the brown-fat lanes (Figure 3B), the signal was much weaker in the brown-fat samples than in the brain samples. Thus, when expressed on a protein basis, the amount of G α 1 was about 150-fold lower in the fractions from control hamsters than in the brain fractions (the exact value is uncertain, as the amount was only slightly above the detection limit by the technique used). The amount of G α 2 was 10-fold lower in the brown-fat fractions than in the brain fractions. However, whereas the brain membrane proteins were unpurified, the brown-fat membranes were purified plasma membranes; assuming a minimal 5-fold purification, it would seem that the plasma-membrane density of G α 1 in brown adipose tissue was about 750-fold lower than in the brain, and that the G α 2 density was about 50-fold lower. This relatively low content of plasma-membrane components is similar to what has earlier been obtained for the amount of Na⁺/K⁺-ATPase, estimated as specific [³H]ouabain-binding capacity [12].

Effect of acclimation to cold on the content of G α proteins

It is clear already from the immunoblot shown in Figure 3A that cold acclimation led to a decrease in the amount of the G α proteins detected. The quantitative and statistical analysis of the densitometric scans of immunoblots of nine independent series of plasma-membrane preparations (control and cold-acclimated samples isolated in parallel) is presented in Table 3.

As seen, the content of both G α 1 and of G α 2 was very significantly reduced, due to cold acclimation, to about 50 % of the control values. There was no significant change in the ratio between these two G α proteins: both in membranes from control

Figure 3 Effect of acclimation to cold on the content of G_{α} proteins in plasma-membrane fractions from brown adipose tissue

(A) Immunoblot. Total membrane preparations from rat cerebral cortex (Br, 40 μ g) and rat glioma C6 BU1 cells (C6, 40 μ g), and purified plasma membranes from brown adipose tissue of control (W, 150 μ g) and cold-acclimated (C, 150 μ g) hamsters, were resolved by SDS/PAGE, electrotransferred to nitrocellulose membranes and immunoblotted as described in the Materials and methods section. The primary antiserum used was SG1 (1:500 dilution). Only the area around 40 kDa (determined with molecular-mass markers) is shown. As discussed in the text, two of the bands may be identified (as indicated) as corresponding to $G_{\alpha 1\alpha}$ and $G_{\alpha 2\alpha}$ peptides. The third band is not found in cortex and only weakly in glioma cells; we refer to this as 'NSP'. (B) Coomassie Blue staining of the polyacrylamide gel after the electrophoretic transfer, demonstrating equal loading of the plasma-membrane fractions from the brown adipose tissue of control and cold-acclimated hamsters.

and from cold-acclimated hamsters the $G_{\alpha 1\alpha}$ content was slightly less than one-tenth of that of $G_{\alpha 2\alpha}$.

It is important to note that we have earlier shown that the level of plasma-membrane markers is actually higher in the membrane fractions from cold-acclimated hamsters than from control hamsters; e.g. the content of Na^+/K^+ -ATPase (estimated as [^3H]ouabain-binding capacity) is increased by $\approx 50\%$ [12]. Thus, expressed per unit of [^3H]ouabain binding, the reduction of $G_{\alpha 1\alpha}$ and $G_{\alpha 2\alpha}$ is even higher than the 50% reduction observed when expressed per mg of protein in Table 3.

It may therefore be concluded that the reduction in the content of G_{α} proteins (both $G_{\alpha 1\alpha}$ and $G_{\alpha 2\alpha}$) in the plasma membranes of brown adipose tissue seen after cold acclimation provides a plausible explanation for the reduced sensitizing effect of pertussis-toxin pretreatment on the respiratory response to noradrenaline in cells from cold-acclimated animals compared with controls (compare Tables 2 and 3).

It was also observed that the content of the so-far-unidentified peptide band NSP was significantly higher in the membranes

Table 3 Effect of acclimation to cold on the content of G_{α} proteins in plasma-membrane fractions from brown adipose tissue

The data are means from nine series of independent plasma-membrane preparations. In each preparation, control and cold-acclimated samples were isolated in parallel and analysed as exemplified in Figure 3. The values are means $\pm$ S.E.M. from nine preparations. *** and (*) indicate significant effects of cold acclimation ($P < 0.001$ and $P < 0.025$ respectively; Student's paired t test); ^{ns}, no significant effect.

	Control	Cold
$G_{\alpha 1\alpha}$ (%)	100	$46 \pm 8^{***}$
$G_{\alpha 2\alpha}$ (%)	100	$57 \pm 4^{***}$
$G_{\alpha 1\alpha}/G_{\alpha 2\alpha}$	0.092 ± 0.013	0.074 ± 0.015^{ns}
NSP (%)	100	$154 \pm 17^{(*)}$

Figure 4 Effect of acclimation to cold on the content of G_{β} subunit in plasma membrane fractions from brown adipose tissue

The experiment was performed principally as described in the legend to Figure 3, except that the antiserum used was BN2 (see the Materials and methods section). Br, 40 μ g of brain total membrane; W and C, 150 μ g of purified plasma membrane from brown adipose tissue of control or cold-acclimated hamsters respectively.

from cold-acclimated animals (154% of the content in the controls). This increase was not quantitatively different from the increase in [^3H]ouabain binding [12], and its physiological significance is therefore difficult to evaluate. It may be noted that the sum of all these immunoreactive proteins ($G_{\alpha 1\alpha} + G_{\alpha 2\alpha} + \text{NSP}$) was significantly reduced as a result of acclimation to cold (to $70 \pm 5\%$; $P < 0.001$). Thus, even if NSP should represent a proteolytic degradation product of a G_{α} -protein, the conclusion that the total content of G_{α} protein was reduced due to cold acclimation would still be valid.

Effect of cold acclimation on the amount of G_{β} -subunits

To evaluate whether the reduction in the content of $G_{\alpha 1\alpha}$ and $G_{\alpha 2\alpha}$ reported above is of physiological significance, it may be important to relate the decrease in the amount of G_{α} -proteins to possible changes in the amount of some other signal-transduction components with which the G_{α} -proteins interact. We have here related the decreases in G_{α} content to changes in the amounts of G_{β} subunit and to adenylyl cyclase activity.

In order to measure the amount of the G_{β} subunit(s), we used the BN2 antiserum previously described [19] (Figure 4). As seen, the amount of G_{β} subunit protein was also much lower in brown adipose tissue than in brain (about 50-fold lower, when expressed on a protein basis). In the immunoblot (Figure 4) it is also seen that there is a tendency to a reduction in the amount of G_{β} subunit, due to cold acclimation. Data from the analysis of all the membrane preparations are compiled in Table 4. As seen, the amount of G_{β} was significantly reduced (to 65%), due to cold acclimation. Therefore, when the ratio between $G_{\alpha 1\alpha}$ and G_{β} , or the ratio between $G_{\alpha 2\alpha}$ and G_{β} , was calculated, no significant

Table 4 Effect of cold acclimation on the content of G-protein β -subunits in plasma-membrane fractions from brown adipose tissue

The data are based on analysis of the nine series of independent plasma-membrane preparations described in Table 3, but one apparent outlier was omitted from the calculations. The data are thus means $\pm$ S.E.M. from eight preparations. The G β data were analysed similarly to the G α data. ** Indicates a significant effect of cold acclimation ($P < 0.01$; Student's paired t-test); ^{ns}, no significant effect.

	Control	Cold
G β (%)	100	65 $\pm$ 9**
G β 1 α /G β (%)	100	70 $\pm$ 14 ^{ns}
G β 2 α /G β (%)	100	91 $\pm$ 11 ^{ns}

effect of cold acclimation was observed. We have not here measured the amount of the G γ subunit, but it would seem from the data available that the content of the entire G γ complex is reduced proportionally due to cold acclimation. The interaction of the different G γ subunits with different adenylyl cyclases is in itself complex [42,43], and the ability of the $\beta\gamma$ subunits to interact with different G α proteins makes it difficult to fully predict what the outcome of the reductions of G β 1 α , G β 2 α and G β would be. It is, however, most likely that the combined reductions indicate a diminished ability of the G γ complex to inhibit adenylyl cyclase activity.

As we have earlier estimated the forskolin-stimulated adenylyl cyclase activity of some of the plasma-membrane fractions studied here [12], we have been able to calculate the effect of cold acclimation on the relationship between the amount of G β 1 α and G β 2 α and adenylyl cyclase. We found that cold acclimation reduced the G β 1 α /cyclase ratio to 51 $\pm$ 12% ($n = 6$; $P < 0.01$) and the G β 2 α /cyclase ratio to 56 $\pm$ 7% ($P < 0.002$). Although the estimation of the catalytic unit of adenylyl cyclase as the forskolin-stimulated value is open to discussion, because the G-proteins may influence the forskolin-stimulated activity [44,45], it may still be suggested that the decrease in G α proteins reported here (together with the decrease in the pertussis-toxin-induced hypersensitization) does indicate that the capacity of the G γ pathway to inhibit adenylyl cyclase is reduced in brown-fat cells from cold-acclimated animals. The forskolin-stimulated adenylyl cyclase activity as such is unchanged by cold acclimation [12].

CONCLUSIONS

In the present investigation we have examined the effect of cold acclimation on parameters in brown adipose tissue related to the capacity of the G γ -protein-mediated signal-transduction pathway. Treatment of animals with pertussis toxin *in vivo* induced a dramatic hypersensitization to noradrenaline in isolated brown-fat cells. This finding indicated a significant role of a pertussis-toxin-sensitive pathway in the adrenergic response of the brown-fat cells. We found that this ability of pertussis toxin to hypersensitize brown-fat cells was reduced by cold acclimation. This decrease in pertussis-toxin effect could be explained by a decrease in the content of G β 1 α and G β 2 α (and G β) proteins in plasma membranes from brown adipose tissue.

It was thus clear from the experimental results that an increased capacity of the receptor/adenylyl cyclase inhibitory pathways could not be involved in the desensitization to adrenergic stimulation observed in isolated brown fat cells after cold acclimation of rodents (and thus after prolonged exposure of the cells to adrenergic stimulation *in situ*).

The findings would rather indicate a decreased ability to transmit inhibitory signals to the adenylyl cyclase. In this context it is interesting to note that we have earlier reported a decreased sensitivity (in reality a full loss of sensitivity) to the inhibitory effect of adenosine in cells from cold-acclimated hamsters, occurring without a decrease in the number of A $_1$ receptors [10]. It has earlier been observed in white adipocytes and in heart *in situ* that prolonged incubation with adenosine analogues leads to effects on G β 1 α , G β 2 α and G β content [19,37,39,46] that are very similar to those reported here. Thus a likely explanation for the present results would be that the chronic adrenergic stimulation of the tissue in the cold-exposed hamster leads (among many other events) to an increased local production of adenosine. This adenosine then chronically stimulates the A $_1$ receptors and through this induces desensitization, by a reduction in the content of the G γ proteins involved in the transfer of the inhibitory signal to the adenylyl cyclase. In this respect, the situation in the brown adipose tissue of cold-acclimated hamsters may adhere to a new, general picture of down-regulation of G-proteins, leading to a desensitized response despite unchanged receptor level, other examples of which have recently been published [47].

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