Down-regulation of $\beta_3$ Adrenoreceptor Gene Expression in Brown Fat Cells Is Transient and Recovery Is Dependent upon a Short-lived Protein Factor*

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The regulation of the expression of the $\beta_3$ adrenoreceptor gene was examined in the brown adipose tissue of intact mice and in murine brown fat primary cell cultures. Both in vivo and in vitro, high levels of $\beta_3$ receptor mRNA were observed. Acute cold exposure of mice resulted in a marked and rapid down-regulation of $\beta_3$ gene expression; this down-regulation was, however, transient. Similarly, in brown fat cell cultures, norepinephrine addition led to down-regulation of $\beta_3$ gene expression, with a lag phase of 30 min and with an apparent half-life of $\beta_3$ mRNA of ~30 min. This down-regulation was stimulated via the $\beta_3$ receptors themselves and mediated via cAMP; the apparent affinity of norepinephrine was extremely high (<1 nM). The degradation rate after actinomycin was identical to that after norepinephrine and was not affected by the presence of norepinephrine; thus, the down-regulation was due to cessation of transcription but not to an increased rate of degradation. Notably, inhibition of protein synthesis by cycloheximide also led to down-regulation. The norepinephrine-induced down-regulation was transient; spontaneous recovery occurred after ~18 h and was not due to depletion of adrenergic agent. Recovery did not occur in the presence of cycloheximide. After recovery, the cells showed a functional desensitization of the down-regulation process itself (EC$_{50}$ now ~10 nM). It is concluded that a down-regulated state cannot explain the functional desensitization of $\beta_3$ adrenergic responsiveness observed in brown fat cells isolated from cold-acclimated animals (i.e. physiologically chronically adrenergically stimulated brown fat cells); since the $\beta_3$ receptor is not subject to desensitization via phosphorylation processes, no satisfactory explanation for the functional desensitization exists as yet. A model is presented for the down-regulation/recovery process, involving the participation of a phosphorylatable short-lived transcription factor.

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Exposure of cells to hormones to which they are responsive often leads to a desensitization of the receptor system for that hormone (1, 2). This homologous desensitization can occur through receptor phosphorylation, through receptor internalization, or through a down-regulation in the steady-state levels of the mRNA for the receptor, resulting in a decrease in receptor density (2–4). The physiological role of such desensitization is believed to be a modulation (attenuation) of the sensitivity of a responsive tissue to chronic stimulation.

Brown adipose tissue is richly sympathetically innervated, and release of norepinephrine, which occurs e.g. during cold exposure of a mammal, stimulates the brown adipocytes to combust fat and to generate heat (5). This thermogenic process is known to continue as long as the mammal requires extra heat production. Thus, in order to continue to fulfill its physiological function, the tissue must be chronically adrenergically stimulated, and there is evidence that this stimulation indeed continues unabated during prolonged demands for thermogenesis (6). This should imply from the above that the adrenergic receptors in the tissue could become desensitized. Nonetheless, for homeostatic reasons, the heat production must remain elevated. There is thus good reason to postulate that the tissue should remain fully responsive to norepinephrine, despite uninterrupted stimulation by the agonist.

Nonetheless, we and others have shown that the brown adipocytes do indeed show physiologically induced functional desensitization. Thus, brown fat cells isolated from animals that have been exposed to cold for a prolonged period show a decreased responsiveness to norepinephrine in vitro, both with respect to the extent of maximal stimulation of oxygen consumption (heat production) and, more importantly, with respect to the EC$_{50}$ for norepinephrine, which is shifted significantly to higher concentrations (7–13). The molecular mechanism behind this functional desensitization is currently not clarified. The decreased sensitivity to norepinephrine can in part be a postreceptor effect, e.g. resulting from increased phosphodiesterase activity, which would decrease the final level of cyclic AMP generated (12), or a transduction effect, resulting from a decreased content of Gs-$\alpha$-subunits, which would attenuate the coupling between the receptor and the adenyl cyclase system (13). However, it is doubtful that these postreceptor mechanisms can fully explain the reported desensitization. It may therefore be suggested that also the receptor itself is involved in the desensitization process, either by being desensitized or by being down-regulated in brown adipocytes.

Pharmacological studies indicate that the $\beta_3$-receptor subtype responsible for the stimulation of oxygen consumption is exclusively the $\beta_3$ subtype (14). The $\beta_3$ receptor lacks most of the serine/threonine residues that are phosphorylated by the $\beta$-adrenergic receptor kinase and by protein kinase A in the classical process of desensitization (15, 16), and the $\beta_3$ receptor...
should consequently lack this fundamental process (3, 17, 18). An alternative explanation for the functional desensitization could therefore be that the β3 receptor is down-regulated, i.e. that a decreased amount of β3 receptors could be found. Such a process can be observed in certain types of transfected cells, e.g. murine L cells, where a decreased amount of β3 receptors is found after adrenergic stimulation (19). In other transfected cells (hamster CHW cells), little effect is observed (18). Not least for technical reasons, it is unknown whether such a down-regulation process occurs under physiological conditions. Since no selective high affinity antagonist has been available for the β3 receptor, there has been no suitable radioligand for determination of receptor density in direct radioligand binding studies, and this means that the situation is difficult to analyze when several β-receptor subtypes may be found within one tissue, as is the case in brown adipose tissue (20, 21). Some studies of β3 receptor densities in brown adipose tissue have been performed with agonists, but these studies have not addressed the question whether the β3 receptor down-regulation occurs in animals when the tissue is exposed to a chronic adrenergic influence during physiological stimulation (22–26). However, it is possible to investigate to what extent the level of gene expression whether a down-regulation occurs. In short-term studies in intact animals, steady-state levels of mRNA for the β3 adrenergic receptor have previously been determined. It has been found that brief cold exposure or acute treatment with norepinephrine or β3 selective agonists decreases the mRNA level for the β3 receptor (23, 27). However, it has not been investigated whether this process could explain the long term desensitization referred to above.

In an attempt to elucidate what changes would be anticipated in the β3 adrenergic receptor levels after chronic cold exposure or agonist stimulation, we have here analyzed steady-state mRNA levels for the β3 adrenergic receptor both in intact animals and in primary cultures of brown adipocytes, exposed acutely and chronically to adrenergic agents. Our results indicate that a β3 mediated process indeed causes a decrease in β3 mRNA levels in these cells, where the presence of the β3 receptor is due to endogenous expression and not to transfection and vector-driven expression in a cell line. Thus, the down-regulation is apparently a physiologically relevant phenomenon. However, we observed that despite continuous stimulation, the β3 mRNA level gradually spontaneously recovered, to reach control levels within less than 24 h; this recovery process was dependent on protein synthesis. We here forward a model for these events and discuss the complex situation that brown fat cells become functionally desensitized to β3 adrenergic stimulation despite the fact that the β3 receptor lacks the molecular prerequisites for desensitization and despite the absence of a persistent down-regulation process.

**MATERIALS AND METHODS**

**Animals—**Six-week-old male mice (NMRI strain, Eklunds, Stockholm) were kept at +28 °C for at least 7 days with free access to food and water. The animals were thereafter either transferred to +4 °C (cold exposure) or handled in the same way and returned to +28 °C (control) for different times. The animals were killed by CO2, followed by decapitation, and the interscapular brown adipose tissue was removed. Homogenization with Ultraspec (Biotecx) was performed with a Potter-Elvehjem homogenizer with a tightly fitting Teflon pestle (10–15 strokes), and total RNA was isolated according to the manufacturer's total RNA isolation method.

**Cell Culture—**Brown fat precursor cells were isolated in principle as described previously (28) from 3-week-old mice that had been kept at the institute at 22 °C for 2–3 days before dissection. The mice were of the same NMRI outbred strain as above. The interscapular, axillary, and cervical brown adipose tissue depots were dissected under sterile conditions. The tissue was carefully minced and transferred to the Hepes-buffered solution (pH 7.4) detailed by Nechad et al. (29), containing 0.2% (w/v) crude collagenase type II (Sigma). Routinely, pooled tissue from six animals was digested in 10 ml of the Hepes-buffered solution. The tissue was digested for 30 min at 37 °C and vortexed every 5 min. The digest was poured through a 250-μm silk filter into sterile tubes. The solution was then put on ice for 15 min to allow the mature brown fat cells and lipid droplets to float. The infranatant was filtered through a 25-μm silk filter and collected in 10-ml sterile tubes. The precursor cells were collected by centrifugation for 10 min at 700 × g, resuspended in Dulbecco's modified Eagle's medium, and recentrifuged. The pellet was resuspended in a volume corresponding to 0.5 ml of cell culture medium for each mouse dissected.

The cell culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Flow), 4 mM insulin, 10 mM Hepes and 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate per ml (30). Aliquots of 0.5 ml of cell suspension were cultivated in 25-cm2 tissue culture flasks (Bibby) with 4.5 ml of cell culture medium, or 0.2 ml of cell suspension were cultivated in six-well culture dishes (Corning) with 1.8 ml of cell culture medium in each well. The cultures were placed at 37 °C in a water-saturated atmosphere of 8% CO2 in air, in a Heraeus CO2-auto-zero B5061 incubator. On days 1 and 3, the cells were washed with prewarmed Dulbecco's modified Eagle's medium, and the medium was changed. Most experiments were performed with cells after 6–7 days in culture, i.e. at confluence. Detailed protocols are found in the description of each experiment.

**RNA Isolation—**At the end of each experiment, the medium was discarded, the cells were dissolved in 800 μl of an Ultraspec (Biotecx) solution, and the manufacturer's procedure for RNA isolation was followed. The final pellet was suspended in 75 μl of 10 mM EDTA, and the RNA was extracted at 70 °C for 15 min with vortexing every second min. RNA concentration was measured, and the absence of protein contamination was checked on a Beckman DU 50 spectrophotometer with readings at 260 and 280 nm. The ratio of 260/280 nm was routinely higher than 1.7.

**Northern Blot—**The solution containing RNA and 10 mM EDTA was lyophilized in a SpeedVac. The RNA was then dissolved in 17 μl of RNA mixture consisting of 50% (v/v) formamide, 0.02× MOPS,1 and 9% (v/v) formaldehyde and 3 μl of loading buffer consisting of 50% (w/v) glycerol and 0.1 mg/ml bromphenol blue. The solution was incubated for 5 min at 70 °C and then chilled on ice. The samples were loaded on a gel (1.25% agarose, 20 mM MOPS, 6.2% (v/v) formaldehyde, and 15 μl of 1 mg/ml ethidium bromide). The gel was run in 20 mM MOPS buffer for 2–3 h at 4–5 V/cm. After electrophoresis, it was verified under UV light from the intensity of the 18 to 28 S RNA bands that all samples were equally loaded and that no degradation was observable.

**Hybridization—**A gel was blotted from the gel to a Hybond-N membrane overnight in 20 × SSC. Three sheets of Whatman 3MM soaked in 20 × SSC were placed on top of the Hybond-N membrane. The gel and the Hybond-N membrane were examined under UV light. The RNA was cross-linked to the Hybond-N membrane (UV Stratalinker 1800 (Stratagene) with the auto-cross-link program).

The Hybond-N membrane was prehybridized with 10 ml of a solution containing 5 × SSC, 5 × Denhardt’s solution, 0.5% SDS, 50 mM sodium phosphate, 50% formamide, and 100 μg/ml of degraded DNA from herring sperm (Sigma) in a hybridization oven (Hybaid) at 45 °C for 3 h. After prehybridization, the Hybond-N membrane was transferred to a similar solution containing the denatured, labeled probe at a concentration of 1–3 × 106 cpm/ml. The hybridization was carried out for at least 16 h at 45 °C. The Hybond-N membrane was then washed twice in 2 × SSC, 0.2% SDS at 30 °C for 20 min each and then twice in 0.1 × SSC, 0.1% SDS at 50 °C for 45 min. The membrane was sealed in a plastic envelope and exposed to a PhosphorImager screen. The screens were analyzed on a Molecular Dynamics PhosphorImager with the ImageQuant program.

**Adrenoceptor cDNA Probe—**The probe originated from the A43 probe earlier characterized (16). A fragment of the mouse β3 adrenoceptor gene was subcloned in pUC18 at the XbaI and SalI site. The plasmid was cut with the restriction enzymes BsmHI and SfiI to give a fragment of 0.5 kilobase pairs.

The probe was labeled with a DNA labeling kit (Boehringer Mannheim) to a length of 0.5 kilobase pairs.

1 The abbreviation used is: MOPS, 4-morpholinepropanesulfonic acid.
**RESULTS**

**Physiological Regulation of \( \beta_3 \) Adrenoreceptor Gene Expression in Intact Animals**—In order to investigate whether the functional desensitization observed in brown adipocytes from cold-acclimated animals involved a down-regulation of the expression of the \( \beta_3 \) adrenoreceptor gene, the effects of acute and chronic cold exposure on \( \beta_3 \) mRNA levels in brown adipose tissue were investigated.

Acute exposure of mice to cold led to a decrease in the levels of mRNA for the \( \beta_3 \) adrenergic receptor in brown adipose tissue (Fig. 1). The level was reduced by as much as 50% after 12 h. This reduction occurs at the same time as a large increase in the level of mRNA for the tissue-specific uncoupling protein can be observed (2). The decrease in \( \beta_3 \) mRNA is depicted in Fig. 3. The cellswere maintained depressedor at least 6 h.

In Fig. 2B, quantitative analyses of the results from a series of similar experiments are depicted. It is clear that there was a lag phase of approximately 30 min, after which there was a very rapid decrease in \( \beta_3 \) mRNA levels. In Fig. 2C, the relevant data from Fig. 2B are plotted on a semilogarithmic scale. The disappearance reasonably followed first-order kinetics; the half-life of \( \beta_3 \) mRNA after norepinephrine stimulation of the cells was as short as 33 ± 2 min.

**Effect of Norepinephrine Dose on \( \beta_3 \) Receptor mRNA Levels**—A dose-response curve for the norepinephrine-stimulated decrease in \( \beta_3 \) mRNA levels is depicted in Fig. 3. The cells were treated for 2 h with different concentrations of norepinephrine and then harvested. It is clear from this curve that already
nanomolar concentrations of norepinephrine were effective in inducing the down-regulation. The calculated EC50 value was remarkably low, 0.7 nM. No other reported action of norepinephrine on brown fat cells has demonstrated such a high apparent affinity; the EC50 for stimulation of the expression of the gene for the uncoupling protein in these cells under identical conditions was \(10\) nM (28), and that for norepinephrine stimulation of cell proliferation was \(20\) nM (39). Heat production (80 nM (14)) and cAMP accumulation (1000 nM (8, 12)) in isolated mature brown fat cells show much higher EC50 values for norepinephrine.

Characterization of the Adrenergic Receptor Involved—The nature of the adrenergic receptor responsible for the rapid decrease in \(\beta_3\) receptor mRNA levels was studied with various adrenergic agonists. As seen in Table I, norepinephrine and epinephrine were equally effective in effecting the down-regulation, as was the subtype-nonselective \(\beta\)-agonist isoprenaline. The subtype-nonselective \(\alpha\)-agonist phenylephrine failed to induce a decrease in mRNA. It may also be noted that the subtype-selective \(\alpha\)-adrenergic antagonists prazosin (\(\alpha_1\)) and yohimbine (\(\alpha_2\)) failed to prevent the norepinephrine-induced down-regulation. Thus, the norepinephrine-induced decrease in \(\beta_3\) gene expression is clearly mediated via \(\beta\)-adrenergic receptors.

It may be observed that the subtype-selective \(\beta\)-adrenergic antagonists ICI-89406 (\(\beta_1\)) and ICI-118551 (\(\beta_2\)) failed to prevent the norepinephrine-induced down-regulation. However, the \(\beta_3\)-selective agonist BRL-37344 mimicked the effect of norepinephrine, and CGP-12177 (an absolute \(\beta_3\) agonist (40) in that it is an antagonist on \(\beta_1\) and \(\beta_2\) receptors) also induced the down-regulation. Thus, the norepinephrine-induced decrease in \(\beta_3\) gene expression is clearly mediated via \(\beta_3\)-adrenergic receptors.

Also, the subtype-selective \(\beta\)-adrenergic antagonists ICI-89406 (\(\beta_1\)) and ICI-118551 (\(\beta_2\)) failed to prevent the norepinephrine-induced down-regulation. The sensitivity to norepinephrine of the down-regulation response was remarkably low, 0.7 nM. No other reported action of norepinephrine on brown fat cells has demonstrated such a high apparent affinity; the EC50 for stimulation of the expression of the gene for the uncoupling protein in these cells under identical conditions was \(-10\) nM (28), and that for norepinephrine stimulation of cell proliferation was \(-20\) nM (39). Heat production (80 nM (14)) and cAMP accumulation (1000 nM (8, 12)) in isolated mature brown fat cells show much higher EC50 values for norepinephrine.
**TABLE I**

| Agent                      | Receptors stimulated | \(\beta_3\) mRNA level |
|----------------------------|----------------------|------------------------|
| Control                    | \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 100 ± 0                |
| Norepinephrine             | \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 13 ± 2                 |
| Norepinephrine and prazosin| \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 7 ± 6                  |
| Norepinephrine and yohimbine| \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 5 ± 4                  |
| Norepinephrine and propranolol| \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 12 ± 1                 |
| Norepinephrine and ICI-89406| \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 9 ± 3                  |
| Norepinephrine and ICI-118551| \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 7 ± 0                  |
| Epinephrine                | \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 3 ± 7                  |
| Phenylephrine              | \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 85 ± 2                 |
| Forskolin                  | \(\alpha_1\) \(\alpha_2\) \(\beta_1\) \(\beta_2\) \(\beta_3\) | 1 ± 1                  |
| M; CGP-12177               | \(\beta_1\) \(\beta_2\) \(\beta_3\) | 18 ± 4                 |
| BRL-37344                  | \(\beta_1\) \(\beta_2\) \(\beta_3\) | 103 ± 4                |
| Forskolin                  | [Ca\(^{2+}\)]\(_i\) | 12 ± 3                 |

is so high, this failure of propranolol to prevent the norepinephrine effect is in accordance with the norepinephrine-induced decrease in \(\beta_3\) receptor mRNA levels being fully a \(\beta_3\) receptor-mediated process. Under the experimental conditions of Table I and assuming an apparent affinity of norepinephrine of 0.7 nM and a \(pA_2\) of 5 for propranolol, the receptor would still be 98% stimulated if it adheres to simple Michaelis-Menten kinetics.

Thus, the combined adrenergic agonist and antagonist studies indicate that the \(\beta_3\) receptor down-regulation is mediated via the \(\beta_3\) receptor itself.

Concerning the intracellular mediation of the adrenergic signal, it is seen that the Ca\(^{2+}\) ionophore A23187 failed to induce a decrease in \(\beta_3\) mRNA. However, the adenylyl cyclase activator forskolin, which markedly increases cAMP levels in these brown adipocyte primary cultures (42), repressed the \(\beta_3\) receptor gene expression. Thus, the down-regulation is presumably brought about by increases in intracellular cAMP levels. The implication of these experiments is that the down-regulation process is not dependent on the \(\beta\)-receptor as such (or on receptor occupancy), but only on an increase in cAMP levels.

**Effect of Inhibition of mRNA Synthesis—**The down-regulation induced by norepinephrine could be due to an increase in the rate of degradation of the \(\beta_3\) mRNA, an inhibition of the transcription of the \(\beta_3\) gene, or both. In order to investigate this, the effects of norepinephrine alone were compared with those of the RNA synthesis inhibitor actinomycin D (with or without norepinephrine). Actinomycin was used at a concentration previously shown not to be detrimental to the cells during short term treatment (43, 44). The results of these experiments are shown in Fig. 4. As seen in Fig. 4A, the half-life of the \(\beta_3\) mRNA after norepinephrine addition was in this series 35 min (uncertainty interval of 28–42 min). The half-life after treatment with actinomycin alone was also 35 min (uncertainty interval of 32–38 min) (Fig. 4B); this would in itself indicate that the down-regulation was mainly due to cessation of transcription. This was confirmed by the observation (Fig. 4B) that the presence of norepinephrine did not significantly alter the rate of down-regulation of \(\beta_3\) mRNA (the half-life was then 41 min; uncertainty interval of 40–42 min; i.e. an increased rate of \(\beta_3\) mRNA degradation was not induced by norepinephrine. The most likely interpretation is thus that the effect of norepinephrine is fully due to suppression of transcription of the \(\beta_3\) gene.

**Effect of Inhibition of Protein Synthesis—**In many instances, inhibition of protein synthesis has been shown to lead to increases in mRNA levels (so-called "superinduction"). We have shown this in the brown adipocyte cultures for the c-fos proto-oncogene (42). Also for some receptors, such an adrenergic up-regulation has been reported (45, 46). However, when the brown adipocytes were treated with cycloheximide in the present study (Fig. 4C), the mRNA levels declined markedly (55-min half-life, uncertainty range of 45–65 min). This is in contrast to that which has been observed after cycloheximide treatment of 3T3-F442A cells (47). Thus, in these primary cultures of brown adipocytes, which express this receptor endogenously, synthesis of a protein of very short half-life is apparently required for maintenance of mRNA levels of the \(\beta_3\) receptor.

**Spontaneous Recovery of \(\beta_3\) Gene Expression in Cell Cultures—**When \(\beta_3\) mRNA levels were followed in cultures that were chronically exposed to norepinephrine, it was found that the dramatic down-regulation seen in Fig. 2 was transient. This is exemplified in Fig. 5A. As seen in Fig. 5B, after 10 h of treatment, the \(\beta_3\) mRNA level started to recover, and by 18 h the level had returned to the level of untreated, control cultures of the same age. The control level was thereafter maintained in the treated cultures for at least a further 20 h. This result was principally in accordance with the results of chronic cold exposure in vivo shown in Fig. 1B.

The transient nature of the down-regulation prompted an investigation as to whether the recovery was a consequence of an artefactual or physiological removal of the stimulus or whether recovery would occur although the stimulus was maintained.

**Action of Long-lived Agonists—**When the initial down-regulation was initiated by 0.1 \(\mu\)M CGP-12177 or by 1 \(\mu\)M forskolin, both of which are anticipated to be more long-lived in their action than norepinephrine, the \(\beta_3\) gene expression was again down-regulated, and the up-regulation occurred with approximately the same kinetics as with 0.1 \(\mu\)M norepinephrine (Fig. 6). An additional dose of forskolin added at the start of the recovery phase delayed recovery somewhat, but it nonetheless occurred. Thus, the recovery phenomenon would not seem to be due to agonist depletion.

This could be further confirmed by the following experiment (Fig. 7A). Cells were treated with 0.1 \(\mu\)M CGP-12177 for 24 h. At this time point, recovery had been attained. The medium from these cells was collected and added to a culture that had not previously been exposed to CGP-12177. Down-regulation was observed in these fresh cultures after 2 h and was of the
same magnitude as that seen in the original culture (Fig. 7A). Similar results were obtained with norepinephrine (not shown). Thus, again, the recovery process does not seem to be a result of full depletion of agonist.

When norepinephrine was readded during the recovery phase, a down-regulation similar to the first one was observed, with respect to both rate and extent (Fig. 7B). This down-regulation apparently occurred in the presence of surviving agonist. Taken together with the observation that surviving agonist could induce down-regulation in naive cells (Fig. 7A), these results would be understandable if some depletion in agonist level had occurred, provided that a desensitization of the adrenergic receptor system had also occurred.

Adrenergically Treated Cells Are Desensitized—To investi-
gate this putative desensitization, cell cultures were pretreated for 18 h with 0.1 mM norepinephrine. Thereafter a dose-res-
sponse curve for norepinephrine-induced down-regulation was made and compared with one obtained with control cells (Fig. 8). It is evident that the pretreatment resulted in cells that were clearly desensitized with respect to their ability to show norepinephrine-induced down-regulation of \( \beta_3 \) mRNA levels; in the naive cells, the EC50 value was 0.9 nM, whereas in the pretreated cells the EC50 value had increased to 7.2 nM. This sensitivity is in the order earlier observed in these cells for other responses (28, 39).

Recovery Requires Protein Synthesis—Self-evidently, the re-
covery phase was completely prevented in the presence of ac-
tinomycin (not shown). In addition, the recovery was also com-
pletely prevented in the presence of cycloheximide, demonstrating a requirement for ongoing protein synthesis (Fig. 9). This is in agreement with the finding shown above that the addition of cycloheximide resulted in a rapid decline in mRNA levels. Since no recovery could occur, a pro-
tein of short half-life is apparently required for gene transcription to proceed. This could be a short-lived transcription factor, the nature of which is currently unknown.
In an attempt to address the question of the molecular basis for the physiologically induced functional desensitization of the β₃ adrenergically mediated thermogenic response in brown fat cells, we have examined in the present investigation the effects of physiological stimulation in vivo and of adrenergic stimulation in vitro on β₃ adrenoreceptor gene expression. Although our results concerning the acute effects agree with those of some others in showing down-regulation of the β₃ gene expression, we have observed, both in vivo and in vitro, that a spontaneous recovery occurs in the maintained presence of physiological or pharmacological stimulation. Thus, the observed acute down-regulation cannot explain the functional desensitization earlier reported.

In order to further study the phenomenon of this transient down-regulation, we have used primary cultures of brown adipocytes from mouse. These cells have previously been demonstrated to develop into genuine brown adipocytes, both with respect to their ability to express the uncoupling protein gene upon adrenergic stimulation (28, 32) and to express the β₃ receptor well, observed as β₃ mRNA (34), as β₃-induced increases in cAMP levels (34, 39) and as functional effects of β₃ stimulation (28).

The Decrease in β₃ Adrenoreceptor Gene Expression—As anticipated from the in vivo experiments in mice, adrenergic stimulation rapidly and dramatically decreased mRNA levels for the β₃ adrenergic receptor in cultured murine brown fat cells; this decrease was clearly mediated through the β₃ receptors themselves and through an elevation in cAMP levels (34, 39) and as functional effects of β₃ stimulation (28).

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Fig. 6. Influence of long acting adrenergic agents on β₃-receptor mRNA levels in cultures of brown adipocytes. Cells that had been cultured for 6 days remained untreated (––––) or were treated at time 0 with 0.1 μM CGP-12177 (CGP) (A) or 1 μM forskolin (For) (B) or with norepinephrine (NE) and harvested at the indicated times for RNA isolation. In the forskolin + forskolin (B) curve, 1 μM forskolin was readded at 7 h. The result shown is from one representative experiment performed in duplicate.

Fig. 7. Demonstration of the presence of CGP-12177 in the culture medium after 24 h in culture. A, cells cultured for 6 days were treated for 24 h with 0.1 μM CGP-12177 (●–●). The medium from these cells was collected after 24 h and added to parallel, untreated (––) cultures from which the medium had been removed. These cells were harvested after 2 further h (+ CGP medium). In control experiments, medium was collected from untreated cells and added to parallel, untreated cells (+ control medium). B, cells that had been cultured for 6 days were treated at time 0 with 0.1 μM norepinephrine. Norepinephrine was added at 15 h or at later time points up to 34 h. Cells were harvested for RNA isolation at the indicated time points (1 h after the second addition). The result shown is from one experiment performed in duplicate.


**Fig. 8. Dose-response curve for the effect of norepinephrine on β3 receptor mRNA levels in brown adipocytes pretreated with norepinephrine.** After 6 days in culture, cells were treated with 0.1 μM norepinephrine. After a further 18 h, the medium was changed and the indicated concentrations of norepinephrine (NE) were added and the cells were harvested after 2 h for RNA isolation (NE pretreatment; ●—●). The open circles indicate the results of a parallel culture treated for 2 h with the indicated concentrations of norepinephrine (no pretreatment; ○—○). The results shown are of one experiment performed in duplicate. The results were analyzed as in Fig. 3.

**Fig. 9. Effect of cycloheximide on the recovery phase during norepinephrine treatment of brown adipocytes in culture.** Cells were cultured for 6 days and then treated with 0.1 μM norepinephrine and 50 μM cycloheximide for the times indicated (●—●). The results shown are of one experiment performed in duplicate. For clarity, results from one of the experiments in Fig. 5 are superimposed on the figure. ●—●, with norepinephrine alone; ○—○, control.

no agonist-induced down-regulation. Thus, it has been of importance to verify in a well studied physiological system (murine brown fat primary cultures) whether or not the gene of the physiologically predominant receptor in that system, the β3 receptor, is down-regulated by adrenergic stimulation. This was clearly the case. Further analysis of the down-regulation process showed several interesting characteristics. The down-regulation had an extremely high sensitivity to norepinephrine (0.7 nM), the highest reported for any adrenergic system in brown adipose tissue and also a much higher sensitivity than any other earlier reported for the β3 receptor mRNA when down-regulated by isoprenaline in 3T3-F442A cells (37), about 4 h for the same receptor mRNA down-regulated by β-agonists in a similar study (38), 10 min when down-regulated by insulin in 3T3-F442A cells (39), 60 min when down-regulated by isoprenaline in murine L cells (19), and 50 min for the β3 receptor mRNA when down-regulated by epinephrine in DDT1 MF-2 cells (50).

A decrease in β3 mRNA was of course also induced by transcriptional inhibition with actinomycin. The degradation rate was identical to that observed after norepinephrine. Thus, an inhibition of transcription would be sufficient to explain the down-regulation; a decreased stability of the β3 mRNA was not induced by norepinephrine.

Interestingly and unexpectedly, down-regulation was also induced by the protein synthesis inhibitor cycloheximide, with a half-life (~55 min) longer than that observed after norepinephrine stimulation. This could indicate that a protein (possibly a transcription factor) with a rapid turnover is necessary for continued transcription of the β3 gene in this cellular environment.

**The Transient Nature of the Down-regulation—** In our murine brown fat cell cultures, the rapid down-regulation induced by norepinephrine proved to be only transient: after about 18 h, the levels of β3 receptor mRNA had returned to control levels (Fig. 5). This phenomenon was in agreement with our observations in mice in vivo (Fig. 1). In both cell cultures and in the animals, we found no agonist-induced increase above the control levels of β3 mRNA, even when the agonist was present for up to about 40 h, although such a long term up-regulation has been reported to occur in 3T3-F442A cells (48).

Although this observation of spontaneous recovery of gene expression is important for the analysis of the β3 desensitization phenomenon, and although similar data indicating an almost total disappearance of β3 transcript and full spontaneous recovery have not been reported earlier for the β3 receptor, the observation of such recovery of receptor expression after down-regulation is not unique. With respect to adrenergic receptors, a similar transient decrease was reported for α1B adrenergic receptor mRNA in smooth muscle cells stimulated by norepinephrine via α1 receptors (51, 54). Hough and Chuang (51) also reported a transient decrease in β2 receptor mRNA levels. Similarly, mRNA levels for both the angiotensin II receptor and the novel vascular smooth muscle receptor decrease and then subsequently increase following a challenge by cyclic AMP elevating agents (55, 56). Also endothelin-B receptor mRNA levels decrease only transiently in response to stimulation of the relevant second messenger pathways (Ca/protein kinase C) (57).

We have demonstrated that the recovery is not caused by depletion of agonist (Fig. 7). Rather, the recovery process could perhaps be understood as being an obligatory event, inevitably occurring after down-regulation (see below). The recovery proc-
Physiologically, a possible “advantage” of the β3 receptor was early suggested to be its probable resistance to short term desensitization because of the particular sequences of the third intracellular loop and C-terminal sequence (18, 19, 37, 48, 60). However, this resistance to one process of functional desensitization was apparently nullified by the down-regulation reported to take place in experiments of an acute nature (27). Although this down-regulation could apparently explain the functional desensitization observed in cells from long term cold-acclimated animals, it removed the teleological basis for the desensitization-resistance advantage of the β3 receptor. The present experiments indicate that this down-regulation is transient in nature and that spontaneous recovery occurs. Thus, the β3 receptor still seems to possess properties making it well suited to mediate prolonged adrenergic stimulation, but it would seem unlikely that β3 receptor-related mechanisms contribute to the functional desensitization observed after physiologically induced chronic adrenergic stimulation of brown fat cells in situ (7–15).

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