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Inhibitory effects of halothane on the thermogenic pathway in brown adipocytes: localization to adenylyl cyclase and mitochondrial fatty acid oxidation

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Abstract

Volatile anesthetics such as halothane efficiently inhibit nonshivering thermogenesis as well as the cellular manifestation of that phenomenon: norepinephrine-induced respiration in brown adipocytes. To identify the molecular site(s) of action of such anesthetics, we have examined the effect of halothane on the sequential intracellular steps from the interaction of norepinephrine with isolated brown adipocytes to the stimulation of mitochondrial respiration (=thermogenesis). We did not identify an inhibition at the level of the adrenergic receptors, but a first site of inhibition was identified as the generation of cAMP by adenylyl cyclase; this led to inhibition of norepinephrine-induced expression of the uncoupling protein-1 (UCP1) gene and reduced norepinephrine-induced lipolysis as secondary effects. Although an inhibition of lipolysis in itself would inhibit thermogenesis, circumvention of this inhibition revealed that a second, postlipolytic, site of inhibition existed: halothane also inhibited the stimulatory effect of exogenous fatty acids on cellular respiration. This inhibition was independent of the presence of UCP1 in the mitochondria of the cells and was thus not directly on the thermogenic uncoupling mechanism. Since not only fatty acid oxidation but also pyruvate oxidation were inhibited by halothane in isolated mitochondria, whereas glycerol-3-phosphate oxidation was not, the second site of action of halothane, evident when cyclase/lipolytic inhibition was circumvented, was located to the respiratory chain, complex I. The results thus explain the inhibition of nonshivering thermogenesis by identifying two sites of action of halothane in brown adipocytes. In addition, the results may open for new formulations of the molecular background to anesthesia.

Keywords: Halothane; Volatile anesthetics; Thermogenesis; Adenylyl cyclase; Fatty acid oxidation; Brown adipose tissue

1. Introduction

The cellular and molecular mode of action of anesthetics is not presently known. Their mode of action can be studied in target systems clearly relevant for anesthesia, i.e. parts of the nervous system, or in systems in which the direct connection between the effects observed and the anesthetic action of the compound is less evident. One such system is brown adipose tissue and thus the related phenomenon of nonshivering thermogenesis.

In human infants (who possess active brown adipose tissue), the rate of oxygen consumption (thermogenesis) fails to increase when the infants are exposed to a cold stress during anesthesia [1]. Similarly, an inhibition of cold-induced nonshivering thermogenesis is seen in halothane-anesthetized rabbits [2]. However, from such results, it is not possible to distinguish whether the inhibition results from halothane effects on the central control of temperature regulation or if the inhibition is on the

Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; UCP1, uncoupling protein 1

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peripheral effector. Norepinephrine-induced nonshivering thermogenesis in rats and hamsters is, however, also inhibited by halothane [3], demonstrating a peripheral site of inhibition. Furthermore, this inhibitory effect of halothane is also observable in vitro, as an inhibition of the norepinephrine-induced increase of oxygen consumption (thermogenesis) in brown adipocytes [4]. Indeed, clinically relevant concentrations of halothane lead to about 70% inhibition of the thermogenic response of brown adipocytes [4]. This inhibition is specific for so-called volatile anesthetics (halothane and similar compounds, as well as chloroform and diethyl ether), in contrast both to chemically similar but anesthetically inactive compounds, and to chemically dissimilar anesthetic compounds (barbiturates etc.) [5].

The molecular site of action of inhalation anesthetics (with halothane as the type compound) in brown adipocytes is not presently known. We therefore designed a series of experiments in which we examined the ability of halothane to inhibit the molecular steps leading from the adrenergic receptors to the mitochondrial combustion process in brown adipocytes.

2. Materials and methods

2.1. Isolation of mature brown adipocytes

This study was approved by the Animal Ethics Committee (Stockholm North Region). Syrian hamsters (*Mesocricetus auratus*) bred at The Wenner-Gren Institute (Stockholm University), 9–28 weeks old, females and males, living at 18–20 °C, with a light/dark cycle of 6 h/18 h and with free access to food and water, were used for isolation of mature brown adipocytes. Two or three hamsters were killed by exposure to 100% CO_2 and decapitation, and brown adipose tissue from the interscapular, cervical and axillary depots was dissected out, cleaned from white fat and connective tissue, and pooled. Brown adipocytes were isolated with the collagenase digestion method previously described in detail [4] in Krebs–Ringer phosphate buffer (in mM: 148 Na^+, 6.9 K^+, 1.5 Ca^{2+}, 1.4 Mg^{2+}, 119.5 Cl^-, 1.4 SO_4^{2-}, 5.6 H_2PO^{4-}, 16.8 HPO_4^{2-}, 10 glucose, 10 fructose, plus 4% crude bovine serum albumin). The isolated adipocytes were stored in a small volume of Krebs–Ringer phosphate buffer on ice for experiments on the same day, or at +5 °C for experiments the day after the preparation. In the latter case, the Krebs–Ringer phosphate buffer was renewed before storage, and the cells were washed once by floating in Krebs–Ringer phosphate buffer for 1 h immediately before experiments.

The UCP1-ablated mice used were progeny of those described by Enerbäck et al. [6], bred at the institute. Wild-type mice were C57BL/6 from B&K Universal, Stockholm. Before the experiments, adult (8–10 weeks old) female mice of either strain were acclimated to 24 °C, with a light/dark cycle of 12 h/12 h, for at least 3 weeks, with free access to food and water. Brown adipocytes from mice [7] were obtained with the same collagenase digestion method as used for the hamster cells, the difference being that the mouse cells were kept at room temperature and that the cells were always used for experiments on the day of preparation.

2.2. Cellular oxygen consumption measurements

Preincubations of the isolated brown adipocytes were performed at 37 °C in sealed plastic tubes with a double-needle technique earlier described [4] in Krebs–Ringer bicarbonate buffer, with the following composition in mM: 145 Na^+, 6.0 K^+, 2.9 Ca^{2+}, 1.2 Mg^{2+}, 128 Cl^-, 1.2 SO_4^{2-}, 1.2 HPO_4^{2-}, 25 HCO_3^-, 10 glucose, 10 fructose and 4% fatty-acid-free bovine serum albumin, pH-adjusted to 7.4 with HCl (the buffer was purchased sterile-filtered (Statens veterinärmedicinska anstalt, Uppsala) with 2% albumin and supplemented with fatty-acid free bovine serum albumin up to 4%). Where indicated, the buffer was further supplemented with 10 mM pyruvate (Sigma) and/or 50 mM carnitine (Sigma). Halothane (Fluothane®; ICI), 3% or as otherwise indicated, was delivered for 15 min via a conventional vaporiser in a gas consisting of 20% O_2, 75% N_2 and 5% CO_2 (AGA); controls were preincubated with this gas only. The concentration of halothane was measured continuously with a gas monitor (Servo gas monitor 120; Siemens) at the inlet of the preincubation tube; a full and stable effect of halothane is observed after <5 min of preincubation, indicating steady-state conditions [4].

The rate of oxygen consumption of preincubated isolated brown adipocytes was measured polarographically with a Clark-type oxygen probe (4004, Yellow Springs Instrument), calibrated at 37 °C with distilled water and sodium dithionite. About 100 000 preincubated cells were added to the magnetically stirred, thermostated (37 °C) oxygen electrode chamber, and the chamber was closed with an airtight cover. The basal rate of oxygen consumption was determined and additions (as indicated) were made with Hamilton syringes through a small hole in the cover of the oxygen electrode chamber. Additions were made about every second min, i.e. when a steady-state in the rate of oxygen consumption resulting from the prior addition had been reached. Each type of experiment was performed in several cell preparations, on different days. Every cell preparation was tested with norepinephrine before the actual experiment was performed. For calculations, distilled water was considered to contain 217 nmol O_2/ml at 37 °C. The rate of oxygen consumption was recorded with a pen recorder (Linear 200), and the output signal from the oxygen electrode amplifier was collected every 0.5 s by a MacLab/2e (application Chart v 3.5). The MacLab Chart file was transferred to the KaleidaGraph Macintosh application for analysis.

2.3. Cellular cAMP measurements

Hamster brown adipocytes in ≈2 ml of the Krebs–Ringer bicarbonate buffer detailed above, with 160 000–175 000 cells/ml, were preincubated for 15 min with 3% halothane, or only carrier gas for the controls. 1.7 ml of the preincubated cell suspension was added to the oxygen electrode chamber and 0.5 ml was taken for determination of the zero time level of cAMP. Thereafter 1 μM norepinephrine was added to the chamber. After registration of the rate of oxygen consumption for 2 min, another 0.5 ml was taken for cAMP determination. The samples were added to Eppendorf tubes with 1 ml ethanol (95%) and formic acid (0.1%).

In forskolin experiments, the forskolin in DMSO was added 1 min after the zero time sample was taken. After 3 min registration of the rate of oxygen consumption, 0.5 ml was taken for cAMP determination. The samples were added to tubes with 1 ml ethanol and 600 μM theophylline.

The tubes were vortexed and centrifuged for 10 min. The supernatant was transferred to new Eppendorf tubes and the pellet was washed with 0.2 ml ethanol:water, 1:1, which was also added to the supernatant. The samples were dried overnight in a warmed vacuum centrifuge and then stored in −20 °C until analysis was performed. cAMP was measured in the dried samples according to the protocol for the Amersham cyclic AMP [^{3}H] assay system.

2.4. Glycerol release determinations

Incubations for glycerol determinations were performed in plastic scintillation vials (25 ml) prepared with 5 ml Krebs/Ringer bicarbonate buffer, with or without 1 μM norepinephrine. 0.5 ml of a hamster brown adipocyte suspension with 275 000 cells were added, and a 2.5 ml sample was immediately taken for determination of the zero time level of glycerol in each incubation. The vials were then placed in a plastic box with an airtight cover and with an inlet and outlet. The box was placed in a slowly shaking water bath at 37 °C. To ensure that a steady-state concentration of halothane was reached, the halothane concentration was continuously measured at the outlet. After 60 min, 2.5 ml were sampled for glycerol determination. The samples were put in plastic tubes and were coagulated with 250 μl 35% perchloric acid, pH-adjusted with 187.5 ml K_2CO_2, and vortexed. The solution was transferred to Eppendorf tubes and centrifuged for 10 min. The supernatant was filtered through filter paper type 3, and the samples were then kept at −20 °C until analysed. Determinations of glycerol concentrations were performed according to the protocol for glycerol determination from Boehringer Mannheim. To examine the viability of the cells and the effect of halothane after 60 min incubation, 3 ml of the cell suspension was preincubated in parallel; 1.1 ml from this cell sample was added to the oxygen chamber, and basal and norepinephrine-stimulated rates of oxygen consumption were measured. The responsiveness to norepinephrine and the inhibitory effect of halothane were then still as previously reported [4].

2.5. Adenylyl cyclase measurements

Brown adipose tissue was isolated from six 30-week-old male hamsters that had been kept at 4 °C for 3 weeks. They were killed by exposure to 70% CO_2 and decapitation, and brown adipose tissue was dissected out from the interscapular, cervical and axillary depots. The pooled tissue was kept in a Tris-sucrose buffer (50 mM Tris-Base, 10 mM $MgCl_2$, 0.5 mM ascorbic acid, 1 mM EDTA, 0.1 mM PMSF, 0.25M sucrose) on ice, minced with scissors, homogenised and centrifuged for 10 min at 9200 × g at 2 °C. The lipids on the surface were discarded and the supernatant was filtered through a silk cloth and put in Quick-Seal™ centrifuge tubes (Beckman Instruments) that were sealed and centrifuged for 30 min at 100 000 × g at 2 °C. The pellets were pooled and resuspended in Tris-buffer (as above) without sucrose, homogenised and again put into Quick-Seal™ tubes and centrifuged as before. The pellet was resuspended and homogenised in Tris-buffer without sucrose, filtered through a silk cloth and kept in aliquots at −70 °C. The protein concentration of the membranes were determined according to the Bradford method with albumin as standard and then diluted to 1 mg protein/ml in 25 mM Tris-buffer (pH 7.4) containing 1 mM DTT.

An assay mix (90 μl) consisting of 25 mM Tris-base (pH 7.4) containing 500 μM ATP, 10 mM $MgCl_2$, 10 mM phosphocreatine, 50 units/ml creatine kinase, 1 μM GTP, 1 mM theophylline and 1 mM dithiothreitol (DTT) was preincubated for 5 min at 37 °C with 10 μM CGP-12177 (dissolved in water) or 100 μM forskolin (dissolved in DMSO) or DMSO alone. A 60 μl of the membrane solution was preincubated for 5 min at 37 °C together with 2.5 mM halothane diluted in DMSO, with DMSO as control. The ligand-assay mix was then added to the membranes and incubated for 10 min at 37 °C. The reaction was stopped by boiling for 5 min. Samples were vortexed, cooled on ice and centrifuged for 5 min at 10 600 × g at 4 °C. A 50 μl aliquot was taken for analysis with the cyclic AMP assay system (Amersham Pharmacia Biotech), according to the manufacturer's instructions.

2.6. Culture of brown adipocytes and UCP1 mRNA determinations

Five-week-old male mice (NMRI strain, local supplier Eklunds) living at 20–22 °C were used for isolation of preadipocytes from brown adipose tissue for cell cultures. Brown adipocyte precursor cells were isolated as described [8]. Cells were cultured in Costar six-well plates (with cells obtained from 2.5 animals inoculated per plate, with 2 ml

culture medium per well) at 37 °C in an atmosphere with 8% CO_2. The culture medium was Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% newborn calf serum (Life Technologies), 2.4 nM insulin (Actrapid Human; Novo), 25 μg/ml sodium ascorbate (KEBO), 10 mM Hepes (GIBCO), 4 mM glutamine (GIBCO), 50 IU/ml penicillin and 50 μg/ml streptomycin (GIBCO). The medium was changed on day 1, 3, and 5, and the cells were used for experiments at day 7.

On day 7, halothane (2.5 mM), diluted in DMSO, was added to the wells of a plate with cultured brown adipocytes (DMSO for controls). The plate was flushed with 3% halothane (or carrier gas only, for controls). The plate was placed in a thermostated water bath (37 °C) in a covered plastic box, with inlet and outlet for the gas. A continuous stream of gas containing 3% halothane, or control gas only, was directed through the box; the halothane concentration was measured at the outlet of the box. After 15 min, 1 μM norepinephrine (or water) was added to the wells, and the plate was incubated for additionally 60 min. In parallel, 2 ml freshly isolated mature brown adipocytes, from hamster, in the Krebs–Ringer bicarbonte buffer detailed above, were preincubated in one of the the wells in each plate. 1.1 ml from this cell sample was added to the oxygen chamber, and basal and norepinephrine-stimulated rate of oxygen consumption were measured, to test the viability of the cells and to verify the halothane effect. The responsiveness to norepinephrine and the inhibitory effect of halothane were, even after 75 min incubation, as previously reported [4].

After the incubation, the culture medium was discarded and the cells were dissolved in 1 ml Ultraspec™ (Biotecx Laboratories) and the manufacturer's procedure for RNA isolation was followed. Northern blot analyses and hybridisation, with a cDNA clone corresponding to mouse UCP1, were performed as previously described [9]. The screen was analysed on a Molecular Dynamics PhosphorImager, and UCP1 mRNA was quantified with the ImageQuant program.

2.7. Mitochondrial oxygen consumption measurements

Cold-acclimated hamsters (14–17 weeks old and kept at 4 °C for 3 weeks before the experiments) were used for preparation of mitochondria from brown adipose tissue. Brown adipose tissue was excised from one hamster. The tissue was kept in 0.25 M sucrose. Mitochondria were isolated by differential centrifugation of homogenised tissue at 0–4 °C [10]. The mitochondria were washed once with 0.2% albumin (Albumin, Fraction V, fatty acid free, Boehringer Mannheim) in 100 mM KCl/20 mM TES (*K-N-tris*(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Sigma) prior to the final wash, and were stored on ice in a small volume of 100 mM KCl/20 mM TES. Protein content was estimated with the fluorogenic reagent fluorescamine (Fluram, Roche), with albumin as standard. 0.2 mg/ml mitochondrial protein was added to an oxygen electrode chamber (at 37 °C) containing 1 ml of a medium consisting of (in mM): 100 KCl, 20 TES, 1 EDTA, 4 $P_{(i)}$, 2 $MgCl_2$ and 3 malate (Sigma) plus 0.1% fatty-acid-free albumin; pH was adjusted to 7.2 with KOH. The mitochondrial preparations were tested in separate experiments for integrity, i.e. we demonstrated that they could be coupled by addition of GDP, as expected for brown-fat mitochondria (not shown). In the experiments where palmitoyl-CoA and palmitoyl-carnitine were added, the medium was supplemented with 5 mM carnitine; this 'extra' carnitine was found in pilot experiments to enhance the oxygen consumption, probably due to endogenous carnitine being lost during the preparation of the mitochondria. To the medium was added 100 μl of a halothane-saturated solution of the above medium, made by incubating 2 ml halothane and 2 ml medium for several hours with shaking at 30 °C. The air phase above the halothane-saturated medium phase (that is on top of the halothane phase in this system) has been determined to contain 35% halothane (E.-B. Kayser, unpublished obs.), and the final halothane concentration in the oxygen electrode chamber therefore corresponded to 3% halothane. Control experiments demonstrated that the inhibition of cellular thermogenesis observed with this method was equivalent with that observed with the preincubation method routinely used for cells (not shown). To control experiments, 100 μl of a similarly prepared medium without halothane was added.

2.8. Chemicals

For experiments with brown adipocytes, the following substances were used: CGP-12177 (Sigma), dissolved in water; norepinephrine ((−)-arterenol bitartrate salt) (Sigma), dissolved and diluted in water; forskolin (Sigma), diluted in DMSO (Sigma) to 20 mM, further dilutions in water; 8-Br-cAMP (8-bromo-3,5-cyclic adenosine monophosphate; Sigma), dissolved and diluted in water; theophylline (1,3-dimethylxanthine; Sigma), oleate (oleic acid sodium salt; Sigma) and palmitate (palmitic acid sodium salt; Sigma) were all dissolved and diluted in hot ethanol:-water, 1:1, in a glas tube, with a fresh solution being prepared for each oxygen trace and kept in solution in a glass tube in a hot water bath; octanoate (caprylic acid; Fluka), diluted in water, pH-adjusted to 7.4 with KOH or NaOH; FCCP (carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Sigma), dissolved in ethanol; paclitaxel (taxol; Sigma), dissolved in ethanol and diluted in water; carnitine and colchicine (Sigma), dissolved in water. Additions of chemicals to the oxygen chamber were made in 3–15 μl volumes with Hamilton syringes.

For experiments with brown-fat mitochondria, the following further substances were used: palmitoyl-L-carnitine (Sigma), oligomycin (Sigma) palmitate (palmitic acid; Calbiochem), all dissolved in ethanol; and palmitoyl-CoA, CoA-SH, ATP, pyruvate and glycerol-3-phosphate, all from Sigma, all dissolved in water.

2.9. Data analysis and statistics

Values are presented as mean ± S.E.M., normalised as described in the figure legends. Bar diagrams were analysed for statistical significance of effect of halothane with Student's paired *t*-test and the signficances expressed in the figures as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Dose–response curves were analysed with a reiterative curve fitting program (KaleidaGraph) for best fit to the equation: $V = \text{basal} + V_{\max}([\text{stimulant}]/([\text{stimulant}] + EC_{50})$ where V is the rate of oxygen consumption, basal is the basal rate of oxygen consumption and $V_{\max}$ is the maximal stimulant-induced rate of oxygen consumption, and EC_{50} is the half maximal effective concentration of the stimulant. Graphs were analysed with two-way ANOVA for effects of halothane (and stimulatory agent concentration) and the significance stated in the legends to the figures.

3. Results

3.1. No involvement of α-adrenoceptors in halothane inhibition of norepinephrine-induced thermogenesis

The increased oxygen consumption (thermogenesis) that can be elicited in brown adipocytes by norepinephrine is attenuated by halothane; 3% halothane inhibits the maximal norepinephrine-induced rate of oxygen consumption by as much as 70% [4]. The most important adrenergic stimulatory pathway for the increase of heat production in brown adipocytes is that mediated via the β_3-subtype of the adrenergic receptors [11] via an increase in cAMP levels and activation of protein kinase A [12]. However, norepinephrine is an agonist on various subtypes of α-adrenergic, as well as on β-adrenergic receptors, and the influence of α-receptors on β-stimulated oxygen consumption is complex. Stimulation of α_2-adrenergic receptors would be expected to lead to a decrease in adenylyl cyclase activity and thus to a decrease in cAMP production and oxygen consumption [13] (however, the presence of α_2-receptors in brown adipocytes specifically from hamsters has been questioned [14]). Furthermore, stimulation of α_1-receptors activates a Ca^{2+}-dependent phosphodiesterase which may decrease cAMP [15] and thus thermogenesis (α_1-stimulation may, however, also have other effects on thermogenesis [11]). The inhibitory effect of halothane could therefore either be located in the stimulatory β-receptor/G_s protein pathway or in the pathways corresponding to either subtype of the α-adrenergic receptors. To investigate whether α-adrenergic stimulation was necessary to observe the inhibitory effect, the effect of halothane on oxygen consumption stimulated via a selective β_3-receptor agonist, CGP-12177, was studied.

In control cells, successive additions of increasing concentrations of CGP-12177 induced, as expected [16], a dose-dependent increase in the rate of oxygen consumption (Fig. 1). When added to brown adipocytes preincubated with 3% halothane, CGP-12177 also induced an increased rate of oxygen consumption, but the rate was substantially attenuated: the maximal rate of CGP-12177-induced oxygen consumption in halothane-preincubated cells was inhibited by about 70% (Fig. 1). Thus, the response to a selective β_3-adrenergic agonist was inhibited by halothane to the same extent as that to the combined α-/β-agonist norepinephrine [4]. The inhibitory effect of halothane is thus explainable based on halothane's effects on the stimulatory β_3-adrenergic pathway and does not necessitate any halothane-induced (over)activation of inhibitory α_1- or α_2-pathways.

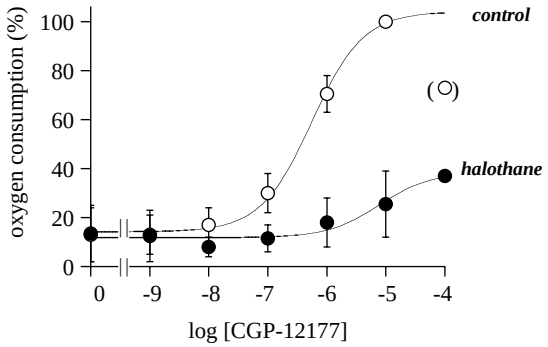

Fig. 1. The effect of halothane on CGP-12177-induced oxygen consumption in isolated brown adipocytes. Isolated brown adipocytes were preincubated for 15 min without halothane ('*control*') or with 3% halothane ('*halothane*'). After preincubation, the cells were immediately transferred to the sealed oxygen electrode chamber, and increasing concentrations of CGP-12177 were successively added to the chamber. The values are the means ± S.E. from two cell preparations (for 10^{-4} from one), normalised to the rate of oxygen consumption with 10 μM CGP-12177 in each adipocyte preparation, 100% corresponding to 270 ± 52 fmol O_2 min^{-1} $cell^{-1}$. Two-way ANOVA based on the mean values shown indicates a significant effect of halothane ($P < 0.05$). Curves are best fits for simple Michaelis–Menten kinetics (omitting the 10^{-4} value for control cells), yielding EC_{50} values of 0.6 μM for control cells and 7 μM for halothane-preincubated cells, with basal values corresponding to 14% respectively 12% of maximal control respiration, and the CGP-12177-induced oxygen consumption being 90 and 27%, respectively (i.e. a reduction in CGP-12177-induced oxygen consumption by 70% due to halothane preincubation).

3.2. Post-receptor level localization of halothane inhibition

As the inhibitory effect of halothane was not due to activation of an inhibitory α-adrenergic pathway, it could be localised to the coupling between the β_3-receptor and adenylyl cyclase. We therefore examined whether the halothane effect could be obviated by circumvention of the coupling process through direct stimulation of adenylyl cyclase with forskolin. Forskolin dose-dependently stimulated oxygen consumption in control cells (Fig. 2A), as expected [17]. In the halothane-preincubated cells, there was also an increase in oxygen consumption, but the response to forskolin was much lower than in the control cells (a 70% decrease) (Fig. 2A). Thus, the halothane inhibition could not be overcome by circumvention of

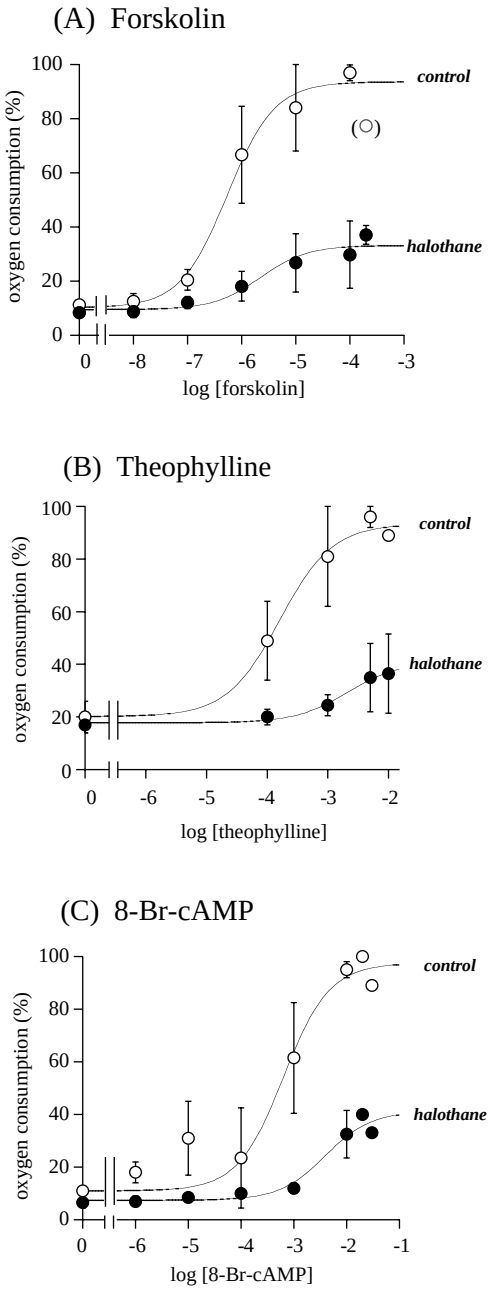

Fig. 2. The effect of halothane on (A) forskolin-, (B) theophylline-, and (C) 8-Br-cAMP-induced oxygen consumption in isolated brown adipocytes. The experiments were performed principally as in Fig. 1. The values are means ± S.E. normalised to the maximal response (irrespective of agonist concentration) in each cell preparation. (A) Forskolin, values from four experiments (100 and 200 μM from two); two-way ANOVA based on the mean values shown indicates a significant effect of halothane ($P < 0.05$); 100% corresponds to 350 ± 75 fmol O_2 min^{-1} $cell^{-1}$. (B) Theophylline, values from three experiments (10 mM from two in halothane and one in control); $P < 0.05$ for halothane effect; 100% corresponds to 288 ± 10 fmol O_2 min^{-1} $cell^{-1}$. (C) 8-Br-cAMP, values from four experiments (20 mM and 30 mM from one); $P < 0.01$ for halothane effect; 100% corresponds to 271 ± 76 fmol O_2 min^{-1} $cell^{-1}$. All curves are best fits to simple Michaelis–Menten kinetics; the relative reduction in stimulant-induced oxygen consumption caused by halothane preincubation was (A) 68%, (B) 70% and (C) 66%.

the receptor/cyclase coupling process and must therefore be located distally to this process.

The level of cAMP is determined not only by adenylyl cyclase activity but also by the activity of phosphodiesterases that degrade cAMP. If phosphodiesterase activity was stimulated by halothane, this could account for the halothane-induced inhibition of oxygen consumption. The effect of a phosphodiesterase inhibitor, theophylline, was therefore examined. Presumably due to an ongoing cAMP production even in non-stimulated cells, theophylline in itself was able to increase oxygen consumption in control cells (Fig. 2B), as previously reported [18,19]. However, even when theophylline was used as the stimulatory agent, oxygen consumption was still much attenuated by halothane (Fig. 2B). Therefore, the halothane effect was not due to phosphodiesterase activation.

To further ascertain that increased cAMP breakdown was not the mechanism for halothane inhibition, we examined the effect of addition of the cAMP analog 8-Br-cAMP, which has greater resistance to phosphodiesterases than cAMP. 8-Br-cAMP dose-dependently induced an increase of oxygen consumption in control cells (Fig. 2C). The maximal rate of oxygen consumption was, however, still much attenuated by halothane, about 65% lower than in control cells (Fig. 2C), confirming that the halothane effect was not located to phosphodiesterase function. This experiments also implies an attenuated thermogenic effect of a given amount of (8-Br)-cAMP.

3.3. Parallel attenuation of adenylyl cyclase activity and reduction of the thermogenic efficacy of cAMP by halothane

The experiment in Fig. 2C indicated that even when identical amounts of 8-Br-cAMP were present in control and halothane-preincubated cells, the increase in oxygen consumption was diminished in the presence of halothane.

To directly determine the effect of halothane on the relationship between a given amount of cAMP and the resulting rate of oxygen consumption, cAMP levels and oxygen consumption were measured simultaneously in cells stimulated with the adenylyl cyclase activator forskolin. For these experiments, forskolin was added as a single dose to a cell suspension (in contrast to successive additions in Fig. 2A), and 3 min later the cAMP level as well as the rate of oxygen consumption were measured. In control cells, cAMP levels increased with increasing forskolin concentrations (Fig. 3A). From a comparison between the cAMP values in Fig. 3A and the corresponding oxygen consumption values in Fig. 3B, it is clear that there was not a linear relationship between cAMP levels and oxygen consumption in the untreated cells; oxygen consumption saturated at a lower forskolin concentration than did increases in cAMP [11]. In Fig. 3C, the relationship between cAMP levels and oxygen consumption is directly depicted. There was basically a positive correlation

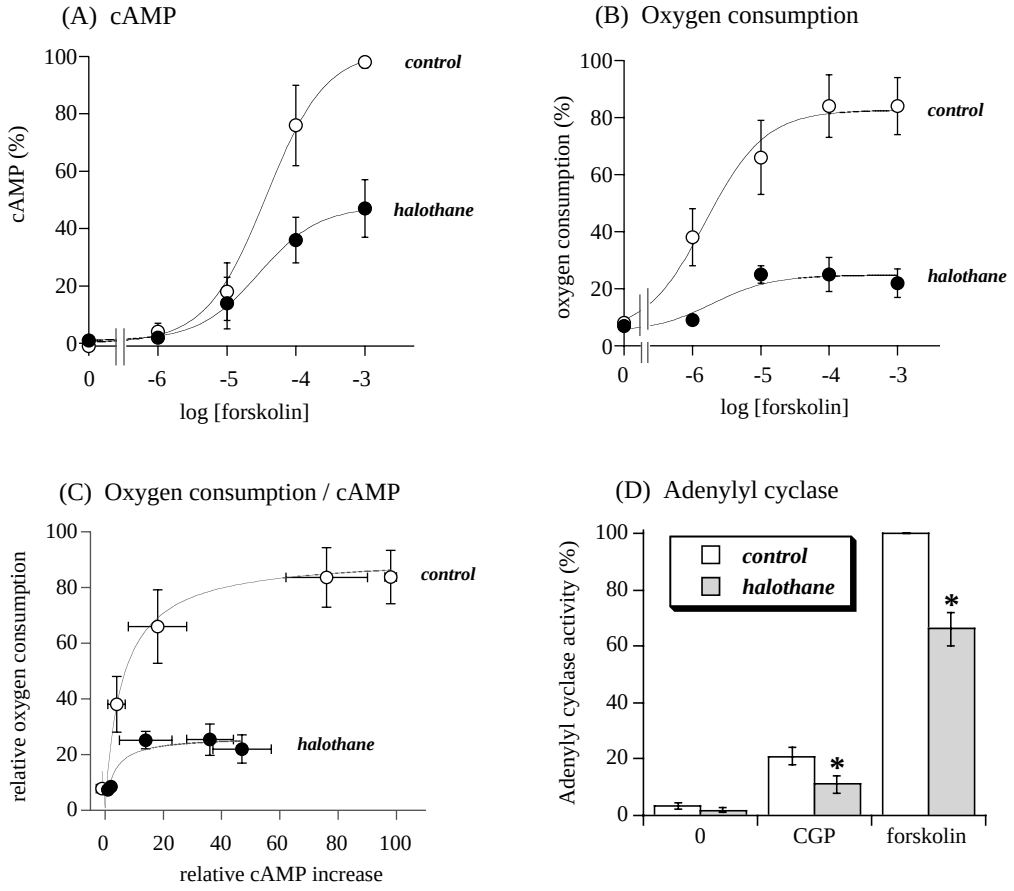

Fig. 3. The effect of halothane on forskolin-induced oxygen consumption, cAMP levels and adenylyl cyclase activity. (A) The effect of halothane on forskolin-induced cAMP levels. After sampling for zero time cAMP levels, a single dose of forskolin in DMSO was added (DMSO alone added for zero concentration), and the experiment otherwise conducted as in Fig. 1 for 3 min after forskolin addition. The values are the differences between the zero time sample and the cAMP amount obtained 3 min after forskolin addition (see Section 2) and are from four to five cell preparations; the cAMP value in the zero-time sample was very low (≈4% of that in 100 μM forskolin-treated cells). In each cell preparation, the highest cAMP level was set to 100%, corresponding to 1060 ± 164 amol cAMP $cell^{-1}$; $P \leq 0.05$ for halothane effect. Curves are drawn for simple Michaelis–Menten kinetics, yielding EC_{50} values of 37 μM for control cells and 29 μM for halothane-preincubated cells, with basal values corresponding to 0.1% respectively 0.9%, and the forskolin-induced cAMP levels being 102 and 47%, respectively (i.e. a reduction in forskolin-induced cAMP levels by 54% due to halothane preincubation). (B) The effect of 3% halothane on forskolin-induced oxygen consumption. The values are from the same experiments as in (A). In each cell preparation, the highest rate of oxygen consumption in controls was set to 100%, and the other values expressed relative to this, presented as means ± S.E. 100% corresponds to 540 ± 63 fmol O_2 min^{-1} $cell^{-1}$; two-way ANOVA based on the mean values shown indicates a significant effect of halothane ($P < 0.05$). Curves are drawn for simple Michaelis–Menten kinetics, yielding EC_{50} values of 1.6 μM for control cells and 2 μM for halothane-preincubated cells, with basal values corresponding to 4.5% respectively 5%, and the forskolin-induced oxygen consumption being 78 and 20%, respectively (i.e. a reduction in forskolin-induced oxygen consumption by 75% due to halothane preincubation). (C) The effect of halothane on the relationship between forskolin-induced cAMP levels and forskolin-induced oxygen consumption. The values are from (A) and (B). Curves are drawn for simple Michaelis–Menten kinetics, yielding 'relative' EC_{50} values for cAMP of 6% for control cells and 3% for halothane-preincubated cells, with basal values corresponding to 0.8% respectively 0%, and the cAMP-induced oxygen consumption being 91 and 27%, respectively (i.e. a reduction in cAMP-induced oxygen consumption by 70% due to halothane preincubation). (D) The effect of halothane on adenylyl cyclase activity in hamster brown adipose tissue cell membranes. Membranes were incubated in the presence of 1 μM CGP-12177 (CGP) or 100 μM forskolin or vehicle, in the absence or presence of halothane. Values are means ± S.E. from three experiments, each performed in triplicate; in each experiment, the cAMP level (adenylyl cyclase activity) observed in forskolin-treated incubations without halothane was set to 100% and the other values expressed relative to this; 100% correspond to 68 ± 9 μmol cAMP observed per mg membrane protein after 10 min incubation; *indicates a significant effect of halothane ($P < 0.05$; Student's paired t-test based on normalised values for each condition).

between the cAMP level and the rate of oxygen consumption, but as cAMP accumulation was strongly induced by forskolin, a plateau was reached where further increases in cAMP did not further increase oxygen consumption (in agreement with [17,11]).

Unexpectedly, also the forskolin-induced cAMP levels were attenuated by halothane (Fig. 3A). The reason that this effect of halothane was unexpected was that the results in Fig. 2 had implied that an inhibition downstream of cAMP would be a sufficient explanation for halothane inhibition of thermogenesis. The experiment in contrast indicated that adenylyl cyclase activity was directly inhibited by halothane. We therefore assayed adenylyl cyclase activity directly in membrane preparations from brown adipose tissue. As seen (Fig. 3D), both CGP-12177 and forskolin increased the cyclase activity in cell membranes from hamster brown adipose tissue; forskolin, as expected, to a much higher degree than CGP-12177. Halothane significantly diminished

the observed cyclase activity, both when the cyclase was stimulated indirectly, via the β_3-receptor, and directly, with forskolin. Thus, halothane had a direct effect on adenylyl cyclase, at least in brown adipose tissue.

The cAMP levels induced by forskolin were lower in halothane-treated cells than in controls. However, when oxygen consumption was plotted as a function of cAMP (Fig. 3C), it was evident that there was not only a decreased cAMP level but also a post-cAMP effect of halothane: in the halothane-preincubated cells, a specific amount of cAMP was not related to the same rate of oxygen consumption as it was in the control cells; the rate was much lower (Fig. 3C) (principally in agreement with the results in Fig. 2C).

Thus, two independent effects of halothane were evident: an inhibition of adenylyl cyclase activity and a lowering of the thermogenic efficacy of cAMP.

3.4. Attenuation of norepinephrine-induced cAMP accumulation and cAMP-dependent cellular events by halothane

From the investigations shown in Fig. 3, it was clear that both β_3-induced and forskolin-induced adenylyl cyclase activities were attenuated by halothane. We therefore ascertained that also cAMP levels induced by the physiological agonist norepinephrine were affected. Norepinephrine increased the cAMP levels in control cells (Fig. 4A), but the actual level obtained with norepinephrine was about 1/30 of that observed with forskolin (33 amol/cell versus 1060 amol/cell (Figs. 3 and 4)). In halothane-treated cells, the increase in cAMP was 40% lower than in control cells (Fig. 4A). Thus, also norepinephrine-induced cAMP accumulation was attenuated by cAMP.

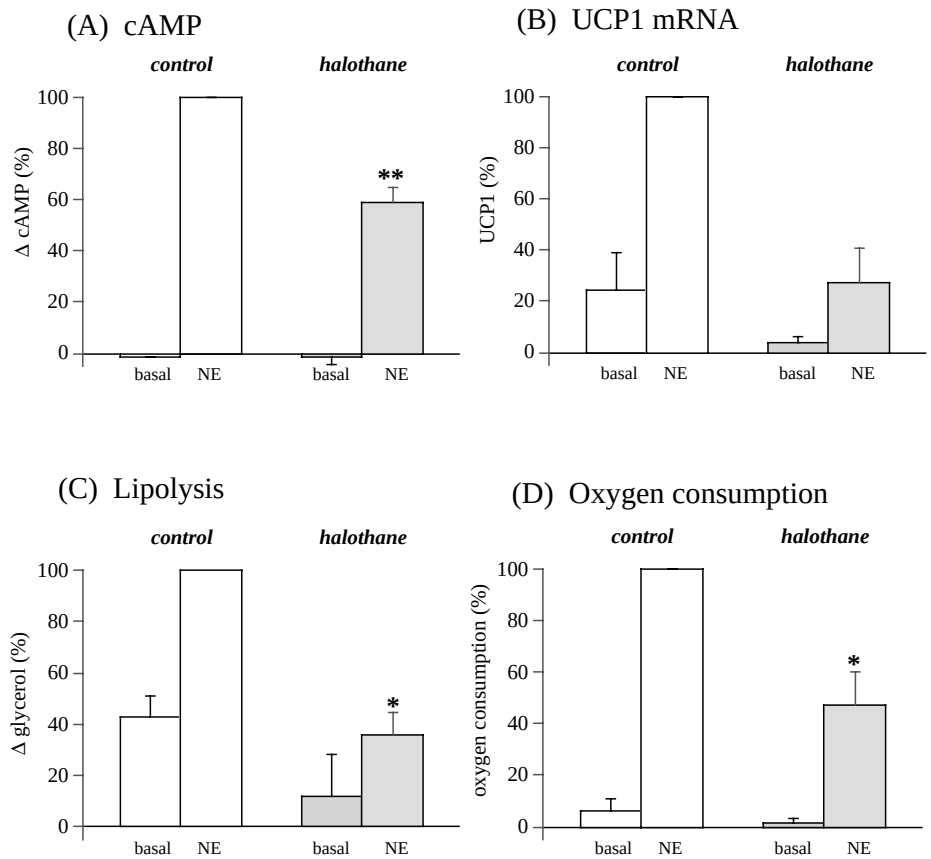

Fig. 4. The effect of halothane on norepinephrine-induced events in brown adipocytes. (A) cAMP levels. Values were obtained as the difference between the zero time cAMP amount in each incubation and the amount at 2 min. The values are the means ± S.E. from four different cell preparations, with duplicates. The cAMP level is normalised to the response to norepinephrine in controls in each cell preparation, 100% corresponding to 33 ± 8 amol cAMP cell^{-1}; ** indicates $P < 0.01$ for halothane effect. (B) UCP1 mRNA levels. Cultured mouse brown adipocytes were preincubated for 75 min with or without 2.5 mM halothane in DMSO [5] (in controls DMSO); 3% halothane, or carrier gas for controls, was continuously added to the atmosphere in the incubation chamber. After 15 min with halothane, 1 μM norepinephrine (water in basal measurements) was added, and the experiment was terminated 60 min later. The values are means ± S.E. from two different cell cultures, based on five to six wells in each culture, normalised to the mean response to norepinephrine in controls in each culture. (C) Lipolysis. Brown adipocytes from hamsters were incubated for 60 min with or without 3% halothane and with or without 1 μM norepinephrine. The values are means ± S.E. from three different cell preparations, with duplicate or singlicate parallel incubations. The increase in glycerol after 60 min is normalised to the response to norepinephrine for controls in each cell preparation, 100% corresponding to 840 ± 174 fmol glycerol cell^{-1}; * indicates $P < 0.05$ for halothane effect. (D) Oxygen consumption. Values are from the same experiments as (A). Basal and norepinephrine-induced (1 μM) oxygen consumption in isolated brown adipocytes was registered 2 min after 15 min preincubation with (or without) 3% halothane. The values are the means ± S.E. from four different cell preparations with duplicates or triplicates in each, normalised to the response to norepinephrine in controls in each cell preparation, 100% corresponding to 384 ± 61 fmol O_2 min^{-1} cell^{-1}; * indicates $P < 0.05$ for halothane effect (Student's paired *t*-test).

As a series of significant processes in brown adipocytes are dependent on cAMP, the implication of the inhibition of cAMP would be that all these processes should be inhibited, as secondary effects. We examined whether this was the case.

Expression of the UCP1 gene is a norepinephrine/cAMP-dependent event in brown adipocytes [20]. Norepinephrine induced a marked increase of UCP1 mRNA in control conditions (Fig. 4B), as expected [20]. The norepinephrine-induced increase of UCP1 mRNA was markedly inhibited by halothane (by 70%) (Fig. 4C).

Lipolysis is another norepinephrine/cAMP-stimulated process and the one that directly leads to thermogenesis, by providing the fatty acids for combustion and UCP1 activation. To evaluate the effect of halothane on lipolysis, glycerol release was measured in suspensions of brown adipocytes. Norepinephrine stimulated lipolysis in control cells, as expected [21] (Fig. 4C). In halothane-incubated samples, norepinephrine practically failed to increase lipolysis (Fig. 4C).

In agreement with this inhibition of lipolysis, it is confirmed in Fig. 4D that also the rate of oxygen consumption was reduced in these halothane-preincubated cells, here by 50% [4]. Thus, the cAMP-dependent processes in brown adipocytes were attenuated by halothane, to at least the extent expected from the halothane inhibition of cAMP accumulation.

3.5. Inhibition of stimulatory effect of free fatty acids by halothane

The direct inhibitory effect of halothane on the norepinephrine-induced adenylyl cyclase activity due to inhibition of lipolysis necessarily leads to thermogenic inhibition. This inhibition may well in reality explain most of the inhibition observed. However, it is also clear that a further site of inhibition must exist. The presence of this site is revealed by the decreased ability of a given amount of cAMP to induce a given amount of thermogenesis (Figs. 2C and 3C). Thus, a further effect, downstream of cAMP, must exist. To identify the nature of the further inhibitory site, we examined the effect of halothane on thermogenesis when cAMP and lipolysis inhibition were circumvented.

Direct addition of free fatty acids to brown adipocytes is sufficient to increase the rate of oxygen consumption [22,23]. If the effect of halothane on norepinephrine-induced thermogenesis in brown adipocytes was solely due to the diminished lipolysis (Fig. 4D), it should therefore be overcome by direct addition of fatty acids.

Oxygen consumption in brown adipocytes was recorded after addition of three different free fatty acids. In control cells, oleate, palmitate and octanoate all stimulated the rate of oxygen consumption in a dose-dependent manner (Fig. 5A–C), as expected. In halothane-preincubated cells, the three fatty acids also all had stimulatory effects, but the experiment revealed a further, post-lipolytic site of inhibition: halothane attenuated the stimulated rates of oxygen consumption by 60–70%, irrespective of which fatty acid was used for stimulation (Fig. 5A–C). Thus, although adenylyl cyclase activity was diminished due to halothane (Fig. 3B) and lipolysis therefore diminished (Fig. 4D), a further site of halothane inhibition existed downstream lipolysis. The existence of this site is only evident when lipolysis inhibition is circumvented.

3.6. No UCP1-dependence of the inhibitory effect of halothane

The fatty acids induce oxygen consumption (heat production) by activation of uncoupling protein-1 (UCP1, thermogenin) in the mitochondria of the cells [7]. If the second site of halothane inhibition downstream lipolysis was the uncoupling process itself, the inhibitory effect should not be visible if the cells were artificially uncoupled, and added fatty acids should then be combusted. However, when the cells were uncoupled by addition of the artificial uncoupler FCCP before the addition of a fatty acid, halothane still inhibited oxygen consumption (Fig. 5D). This indicates that the second site of inhibition was not (mainly) the uncoupling protein or the uncoupling process.

A more direct tool to investigate whether the halothane effect is related to mitochondrial uncoupling are mice without UCP1 [6]. In brown adipocytes from wild-type mice, oleate induced oxygen consumption to such an extent that FCCP was unable to further increase the rate of oxygen consumption (Fig. 6A) [7]. In these brown adipocytes from wild-type mice (as in hamster cells (Fig. 5A–D)), halothane also inhibited the oleate-induced oxygen consumption (Fig. 6A), and the effect could not be overcome by subsequent FCCP stimulation. This is also an indication that the second site of inhibition is not specifically on the UCP1 uncoupling process. In mice without UCP1, oleate was in itself unable to induce thermogenesis, as earlier observed [7]. When the cells were artificially uncoupled by FCCP, the oleate was combusted, but the rate was again diminished by halothane (Fig. 6B).

In cells from wild-type mice (as in hamster cells), norepinephrine was also able to increase the rate of oxygen consumption, and no further stimulation was induced by FCCP (Fig. 6C). Halothane again inhibited the norepinephrine response, and the inhibition could not be overcome with FCCP (Fig. 6C). Norepinephrine did not induce oxygen consumption in cells from UCP1-ablated mice, but the uncoupling effect of FCCP was evident (Fig. 6D); the observed respiration after FCCP is based on substrates (fatty acids) endogenously formed by norepinephrine addition and activation of lipolysis [7]. Halothane, also in this case, inhibited the FCCP-induced respiration (Fig. 6D).

Thus, these experiments indicate that the second site of the halothane effect was related to the catabolic capacity of

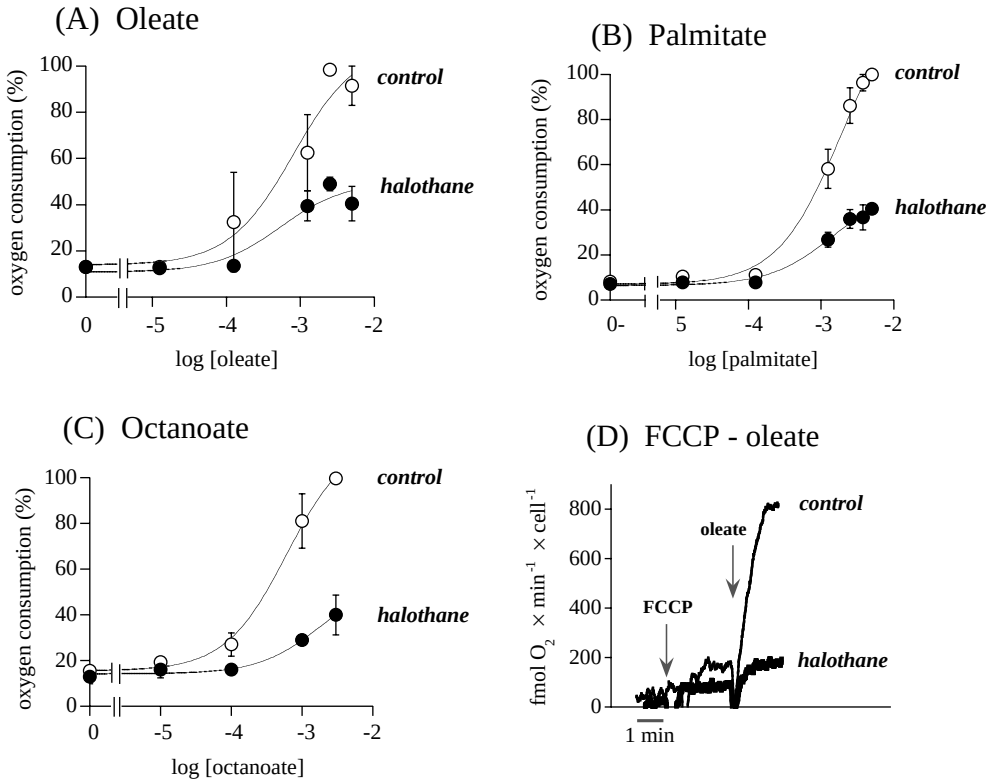

Fig. 5. The effect of halothane on the dose–response curves for fatty acid-induced oxygen consumption in isolated brown adipocytes. Experiments were performed as in Fig. 1 but with the indicated fatty acids as stimulants. The values are means ± S.E. normalised to the maximal response (irrespective of fatty acid concentration) in each cell preparation. (A) Oleate, values from two experiments, 100% corresponding to 524 ± 127 fmol O_2 min^{-1} $cell^{-1}$; two-way ANOVA based on the mean values shown yields a significant effect of halothane ($P < 0.05$). (B) Palmitate, values from four experiments (5 mM value from 1), 100% corresponding to 549 ± 95 fmol O_2 min^{-1} $cell^{-1}$; $P < 0.05$ for halothane effect. (C) Octanoate, values from four experiments (10 μM and 3 mM values from three), 100% corresponding to 258 ± 55 fmol O_2 min^{-1} $cell^{-1}$. (D) Oxygen traces demonstrating the effect of 3% halothane on oxygen consumption observed after the addition of 40 μM FCCP and 5 mM oleate to hamster brown adipocytes.

the mitochondria, rather than to the uncoupling process as such.

3.7. No effect of halothane on transport of fatty acids to the mitochondria

Both mitochondrial uncoupling by fatty acids and combustion of fatty acids demand an intracellular transfer of the fatty acids from the site of lipolysis to the mitochondria. The second inhibitory effect could therefore be related to the processes involved in fatty acid transport within the cells and presentation to the mitochondria. A microtubule-associated transport of lipid droplets in brown adipocytes has been suggested [24]. There are reports of an inhibitory, microtubule-depolymerizing ('colchicine-like') effect of halothane on cytoskeletal components in different cell types [25,26]. The possibility therefore existed that the effect of halothane could be due to an inhibition of such microtubule-dependent lipid transport. The alkaloid taxol (paclitaxel) has a microtubule-stabilising effect [27], and it could be speculated that taxol thus could counteract the halothane effect. We found that cells incubated with 10 μM taxol for 30 min before stimulation with 1 μM norepinephrine responded normally to norepinephrine (not shown). However, also in these taxol-preincubated cells, halothane attenuated the rate of oxygen consumption (not shown). Thus, the inhibitory effect of halothane was probably not due to microtubule depolymerization. Concordingly, we observed that the microtubule-depolymerizing agent colchicine (10 μM, 30 min) did not affect the response to norepinephrine in control cells (in contrast to an inhibitory effect on glucose oxidation earlier observed [28,29]), and halothane still attenuated the rate of oxygen consumption to the same extent as in previous experiments (not shown).

3.8. No effect of halothane on carnitine-dependent transport of fatty acids

Fatty acid oxidation requires carnitine for transport of the fatty acids into the mitochondrial matrix. In an extension of studies reporting an alleviation of halothane effects on isolated liver mitochondria by carnitine supplementation [30,31], we examined whether supplementation of carnitine to the brown adipocytes could make them less sensitive to the inhibitory effect of halothane.

In brown adipocytes preincubated in the normal buffer solution, norepinephrine increased the rate of oxygen

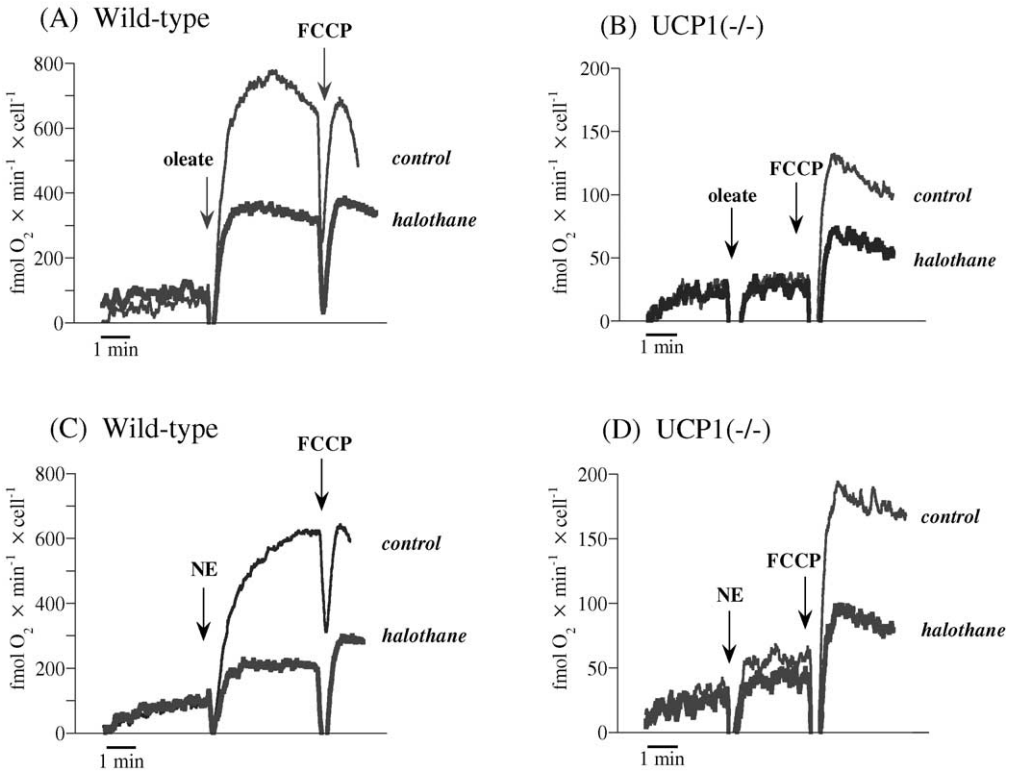

Fig. 6. The effect of halothane on oxygen consumption in brown adipocytes from wild-type and UCP1-ablated mice. Experiments were performed as in Fig. 1. The traces are the running means from duplicate registrations (oleate controls single). Traces show rates of oxygen consumption induced by addition of 5 mM oleate and 40 μM FCCP to brown adipocytes from (A) wild-type mice and (B) from UCP1-ablated mice, as well as rates induced by 1 μM norepinephrine (NE) and 40 μM FCCP in brown adipocytes from (C) wild-type mice and (D) UCP1-ablated mice.

consumption as expected (Fig. 7A), and halothane inhibited the response to norepinephrine (Fig. 7A). When preincubation was performed in this buffer supplemented with carnitine, the stimulatory effect of norepinephrine was similar to that without supplementation, and the inhibitory effect of halothane was also the same (Fig. 7B). Thus,

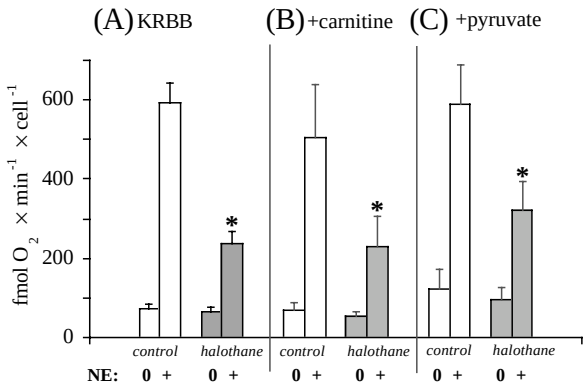

Fig. 7. The effect of halothane on norepinephrine-induced oxygen consumption in isolated brown adipocytes preincubated for 15 min without (*control*) or with 3% halothane (*halothane*) in (A) Krebs–Ringer bicarbonate buffer (KRBB), (B) Krebs–Ringer bicarbonate buffer supplemented with 50 mM carnitine, or (C) Krebs–Ringer bicarbonate buffer supplemented with 10 mM pyruvate. The experiments were performed as in Fig. 1. The values are means ± S.E. from three different cell preparations. * indicates a significant effect of halothane ($P < 0.05$; Student's paired *t*-test).

supplementation of brown adipocytes with carnitine could not counteract the inhibitory effect of halothane on oxygen consumption. The alleviating role of carnitine in isolated mitochondria has been suggested to be caused by prevention of accumulation of inhibitory acyl-CoAs [30,31], and such an accumulation would therefore not seem to be related to the second site of halothane inhibition in brown adipocytes.

3.9. No depletion of metabolic intermediates by halothane

A possibility has been discussed that halothane may interact with the mitochondria within the cells in such a way that the level of citric acid cycle intermediates is affected [32]. If the level of intermediates is diminished, the rate of norepinephrine-induced oxygen consumption is also diminished, as is seen experimentally, e.g. by ammonia treatment of brown adipocytes [33]. However, a replenishment of citric acid cycle intermediates may be obtained through the anaplerotic activity of pyruvate carboxylase [33,34]. A reduction of the level of intermediates by halothane could therefore be a reason for the inhibitory effect of halothane on oxygen consumption. In that case, an excess of pyruvate (in the presence of CO_2 from the buffer) should alleviate the inhibition (as it alleviates ammonia inhibition [33]). Brown adipocytes supplemented with

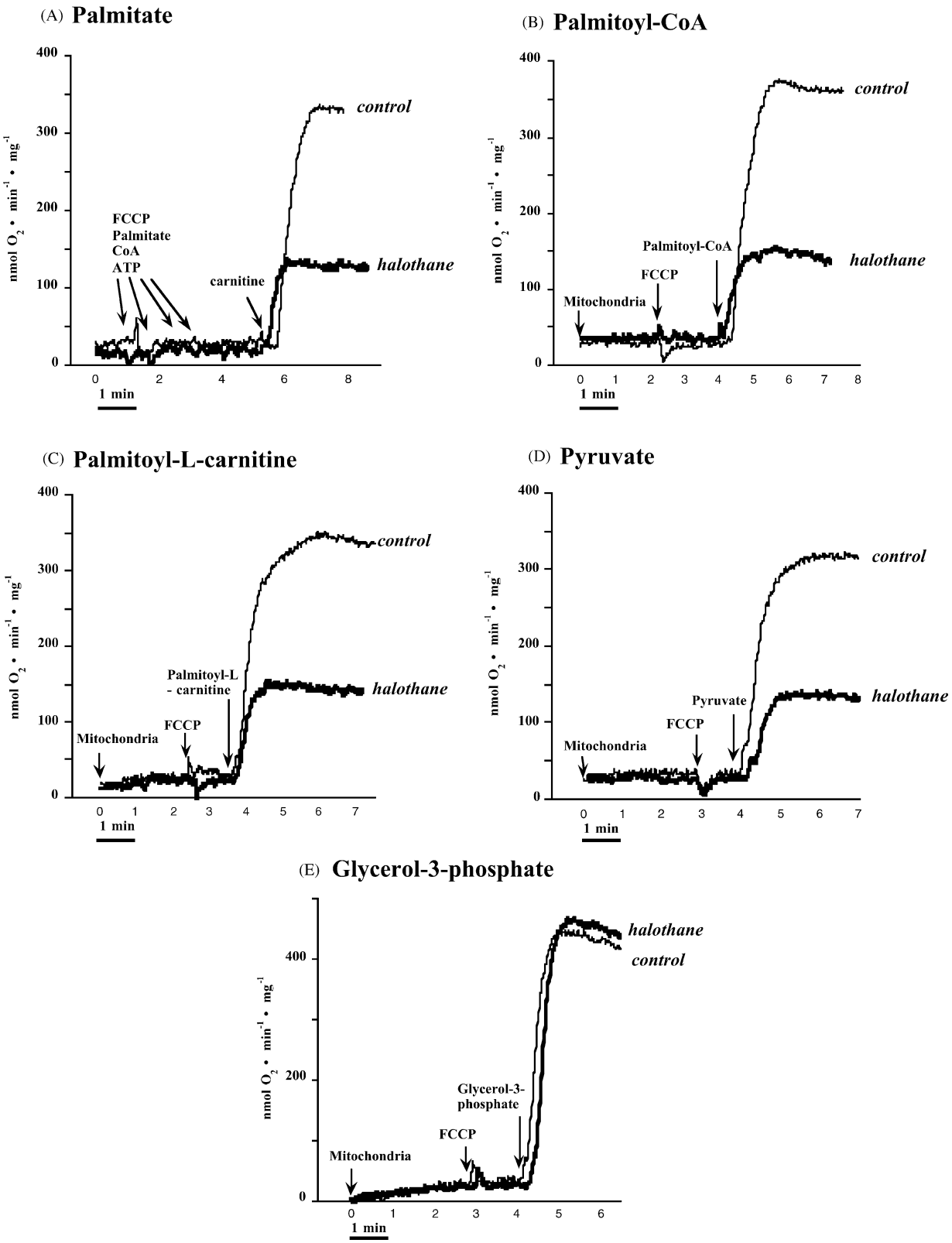

Fig. 8. The effect of halothane on oxygen consumption in mitochondria from brown adipose tissue. The mitochondria were examined in the absence (*control*) or in the presence of halothane (*halothane*) but always in the presence of the uncoupler FCCP (0.7 μM). (A) 0.5 μg/ml oligomycin, 15 μM palmitate, 16 μM CoA-SH, 1.5 mM ATP and finally 5 mM carnitine was added. (B) 30 μM palmitoyl-CoA was added in the presence of 5 mM carnitine. (C) 18 μM palmitoyl-carnitine was added in the presence of 5 mM carnitine. (D) 5 mM pyruvate was added. (E) 5 mM glycerol-3-phosphate was added. Traces are examples from one mitochondrial preparation; in mean from two to five traces from one to four different mitochondrial preparations, halothane inhibited the respiration by (A) 62 ± 4%, (B) 62 ± 5%, (C)58 ± 2%, (D)56 ± 2% and (E)−8 ± 5%.

pyruvate responded to norepinephrine stimulation in the same way as cells incubated without pyruvate (Fig. 7A and C). However, when the buffer was supplemented with pyruvate, halothane inhibited norepinephrine-stimulated oxygen consumption to the same extent as in buffer without pyruvate (Fig. 7A and C). Thus, the second site of halothane inhibition is probably not due to a lack of citric acid cycle intermediates ('condensing partners' for acetyl-CoA from β-oxidation).

3.10. The effect of halothane on brown adipose tissue mitochondrial respiration

For mitochondrial oxidation, fatty acids are first activated to acyl-CoAs which are then converted to acyl-carnitines for transport into the mitochondria. In the mitochondria, acyl-carnitine is reconverted to acyl-CoA and enters β-oxidation. To test if the activation of fatty acids to acyl-CoAs, the transport of acyl-carnitine into the mitochondria, or the mitochondrial combustion itself was inhibited by halothane, experiments were designed to examine the effect of halothane on each of these steps.

When a fatty acid and the components necessary for activation and transport of fatty acids were all added to an uncoupled hamster brown-fat mitochondria preparation, oxygen consumption was, as expected, stimulated (Fig. 8A). In this case, the halothane effect was preserved (Fig. 8A). Thus, the second site of halothane inhibition was located beyond the fatty acid activation step (i.e. acyl-CoA synthesis). Respiration was also induced when the fatty acids were added in the form of acyl-CoA (Fig. 8B). Halothane inhibition of oxygen consumption was preserved (Fig. 8B). Thus, the second site of halothane inhibition was located beyond the acyl-carnitine formation step. Addition of palmitoyl-carnitine to the mitochondria also led to rapid respiration [35] (Fig. 8C), and halothane was still inhibitory. Thus, from these experiments, it was evident that halothane had its second inhibitory site on metabolic steps downstream acyl-carnitine formation (acyl-carnitine transport over the mitochondrial membrane, intramitochondrial acyl-carnitine transferase, β-oxidation etc).

One such step is the respiratory chain itself. In order to examine whether the effect of halothane was specific for acyl-carnitine catabolism or was general for substrate oxidation in these mitochondria, we tested the effect of halothane on two other types of substrate: pyruvate and glycerol-3-phosphate. As seen (Fig. 8D), halothane also inhibited the oxidation of pyruvate, whereas the oxidation of glycerol-3-phosphate was unaffected by halothane (Fig. 8E). Pyruvate, as well as palmitoyl-carnitine, are so-called complex-I-linked substrates for mitochondrial oxidation, whereas glycerol-3-phosphate enters the respiratory chain below complex-I. It can therefore be concluded that halothane's second site of inhibition in brown adipocytes during thermogenesis is located to complex I of the mitochondria.

4. Discussion

In this study, we have attempted to identify the inhibitory site(s) of action of halothane in brown adipocytes. We found that two clearly distinguishable sites could be delineated: the adenylyl cyclase, which functionally must be considered the primary site, and the oxidation of fatty acids in the mitochondria—a site that is revealed when the lipolysis inhibition due to cyclase inhibition is experimentally overcome (Fig. 9). These observations may facilitate the molecular understanding of cellular effects of halothane and other inhalation anesthetics. The possibility may also be discussed that the effects on brown adipocytes can have parallels in the central nervous system and thus be related to the anesthetic effects of these types of compounds.

4.1. The sites of inhibition

We did not observe any direct effect of halothane on the β_3-adrenoceptor or on any other adrenergic receptors in the system investigated here (in contrast to what has been observed in another system [36]). We observed a halothane inhibition of directly stimulated adenylyl cyclase activity (Figs. 2 and 3); this inhibition should be sufficient to explain the diminished cAMP accumulation found after adrenergic receptor stimulation. Direct inhibitory effects of halothane on adenylyl cyclase have not been reported in other systems; however, both stimulatory and inhibitory effects of halothane on basal levels of and receptor-mediated increases in cAMP have been reported [37–40]. The different observations in the different tissues investigated could perhaps be due to the presence of different subtypes of adenylyl cyclase.

The inhibitory effect of halothane on adenylyl cyclase necessarily affects all downstream cAMP-dependent events in the cell (Fig. 9). It would therefore seem that further observations of different sites of inhibition would be trivial. Indeed, norepinephrine-induced UCP1 gene expression and lipolysis, both cAMP-dependent processes, were inhibited by halothane (Fig. 4C and D). Decreased lipolysis has earlier been observed in white adipocytes [41], but the mechanism has not been elucidated.

However, when we circumvented the depression of lipolysis by directly adding fatty acids to the brown adipocytes (Fig. 9), thermogenesis was not restored (Fig. 5A–D). This was a clear demonstration of a further site of inhibition. We found no evidence that the inhibition was located to the steps transferring the fatty acids from cytosol into the mitochondria, but fatty acid oxidation as such was inhibited. As the oxidation of another complex-I-linked substrate (pyruvate), but not of a substrate that enters the respiratory chain below complex-I (glycerol-3-phosphate), was similarly inhibited, there is no reason to assume that the second site of inhibition is located to β-oxidation or citric acid cycle; rather complex I would seem to be the direct target. This second site of inhibition is in agreement with

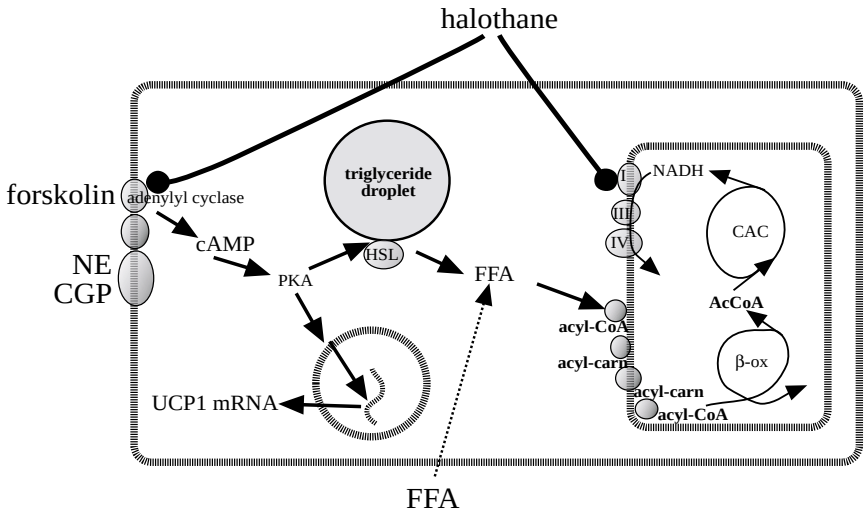

Fig. 9. The two sites of action of halothane in brown adipocytes. Summary figure indicating the processes affected by halothane in brown adipocytes. Halothane inhibits adenylyl cyclase and complex I in the respiratory chain; the latter action is, however, only evident when free fatty acids (FFA) are provided to the cells, distally to the inhibition of adenylyl cyclase (stippled line). NE, norepinephrine; CGP, CGP-12177; PKA, protein kinase A; HSL, hormone-sensitive lipase; β-ox, β-oxidation; CAC, citric acid cycle.

observations of inhibition of complex-I-linked substrate oxidation in mitochondria from other tissues [42,43] and direct and indirect observations of halothane effects on the NADH-ubiquinone oxidoreductase [44,45]. It may therefore be considered a general site of action of halothane which, however, is of less relevance in the brown adipocyte system where the inhibition of adenylyl cyclase would preclude normal activation of the mitochondrial catabolic system.

4.2. A possible relationship between anti-thermogenic and anesthetic effects of halothane

The data and analysis of the effect of halothane on brown adipocytes and thermogenesis may at first sight not be considered relevant for the understanding of the anesthetic effects of halothane. Indeed, although there is a classical interest in metabolic effects of anesthetics [46–48], present contention is that the anesthetic effects are not primarily on metabolism but rather on central neural control. Accordingly, practically all present interest is concentrated to interactions of inhalation anesthetics with transmitters in the central nervous system [49,50]—although as yet, no single transmitter target has been identified.

It may be noted that halothane had only a minor effect on the basal metabolism of the brown adipocytes; the inhibitory effect was predominantly on the norepinephrine-induced increase in metabolic rate. Based on this type of observation, a possible point of view could be that the anti-thermogenic effects analysed here could have parallells in the central nervous system, especially in those regions responsible for arousal and the awake state. It is generally imagined that these centres display a constantly increased activity (‘metabolism’) in the awake mode. If the anesthetics also decrease the induced metabolism (i.e. the general activity) of these centres, it is perhaps possible that the effect that is manifest in brown adipocytes as an anti-thermogenic effect, would be manifest in the central nervous system as a decreased arousal tone, i.e. as a component of anesthesia.

Acknowledgments

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