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α_1-Adrenergic Stimulation Potentiates the Thermogenic Action of β_3-Adrenoreceptor-generated cAMP in Brown Fat Cells*

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The relationship between cAMP levels and thermogenesis was investigated in brown fat cells from Syrian hamsters. Irrespective of whether the selective β_3-, β_2-, and β_1-agonists BRL 37344, salbutamol, and dobutamine or the physiological agonist norepinephrine was used to stimulate the cells, increases in cAMP levels were mediated via the β_3-receptor, as were the thermogenic effects. However, the relationship "thermogenesis per cAMP" was much lower for agents other than norepinephrine. Similarly, forskolin, although more potent than norepinephrine in elevating cAMP, was less potent in inducing thermogenesis. The selective α_1-agonist cirazoline was in itself without effect on cAMP levels or thermogenesis, but when added to forskolin-stimulated cells, potentiated thermogenesis, up to the norepinephrine level, without affecting cAMP. This potentiation could not be inhibited by chelerythrine, but could be mimicked by Ca^{2+} ionophores. It was apparently not mediated via calmodulin-dependent protein kinase and was not an effect on mitochondrial respiratory control. The ability of all cAMP-elevating agents to induce thermogenesis in brown fat cells has earlier been interpreted to mean that it is only through the β-receptors and the resulting increase in cAMP levels that thermogenesis is induced. However, it is here concluded that the thermogenic response to norepinephrine involves two interacting parts, one mediated via β-receptors and cAMP and the other via α_1-receptors and increases in cytosolic Ca^{2+} levels.

It is the generally accepted view (1–7) that the initial steps in the pathway leading from norepinephrine stimulation of brown fat cells to the final thermogenic reaction are quite well understood: through interaction with β-adrenergic receptors and transduction via G_s proteins, norepinephrine activates adenylyl cyclase. The sympathetic stimulation is thus intracellularly mediated by an increase in cAMP levels. cAMP, through its activation of protein kinase A and a subsequent activation of hormone-sensitive lipase, leads to release of free fatty acids from the triglycerides in the cells. The free fatty acids are combusted in the mitochondria; they are thus the substrate for thermogenesis, and they are also, in a less well understood way, involved in the activation of the uncoupling protein UCP1 (see for example, Ref. 8 for review).

It is an inherent implication of this model that it is the cAMP level in the brown fat cells that, during acute thermogenesis, solely determines the final outcome of the acute sympathetic stimulation. Thus, the means by which the cAMP is elevated is implicitly not of interest: any agent that has the ability to increase cAMP levels is also expected to elicit a proportionate increase in thermogenesis.

However, this view has been so generally accepted that no detailed analysis has been presented. We present here data challenging this simple relationship and indicating that both a β_3-receptor-induced increase in cAMP and an increase in Ca^{2+} caused by α_1-adrenergic stimulation may in reality be of significance for the norepinephrine-induced thermogenic process.

The presence of α_1-adrenergic receptors in brown adipose tissue has long been demonstrated (9–11), but until now, the role of α_1-receptors in the acute thermogenic function of the tissue has been considered to be small (12, 13) or nonexistent (14). We have thus here unveiled a hitherto overlooked role of α_1-adrenergic stimulation and confer to the α_1-receptors on brown fat cells a significant role also in the acute thermogenic process.

EXPERIMENTAL PROCEDURES

Isolation of Brown Adipocytes—Brown adipocytes were isolated with the collagenase method described earlier (15). Each preparation was from two adult (10–30-week old) Syrian hamsters (*Mesocricetus auratus*) of either sex. The hamsters had been kept at 20 ± 1 °C, one per cage, with food and water *ad libitum*.

cAMP and Respiratory Rate Determinations—For parallel measurements of cAMP levels and thermogenesis (rate of oxygen consumption), 50,000–80,000 brown fat cells were added to a magnetically stirred oxygen electrode chamber (thermostated at 37 °C) containing 1.1 ml of Krebs-Ringer bicarbonate buffer (see "Buffers") and fitted with a Yellow Springs Model 4004 Clark-type oxygen probe. The suspension was covered with a lid, and the oxygen tension and oxygen consumption rate were continuously monitored. After 4 min, the agent was added with a syringe through a hole in the lid, and the oxygen consumption was followed. After 10 min, the incubation was terminated by taking a 0.5-ml aliquot of the suspension and transferring it to 1 ml of 99.5% ethanol. This suspension was dried in a Speedvac centrifuge for 12 h at room temperature. The dried samples were dissolved in 200 μl of buffer 1 provided with the [^{3}H]cAMP assay system from Amersham Corp. and centrifuged in an Eppendorf centrifuge at 12,000 rpm for 12 min. Two 50-μl aliquots of the supernatants were analyzed according to the description provided with the assay system.

In certain experiments, only oxygen consumption was followed in the same experimental system. In certain experiments, only cAMP levels were followed in the same experimental system, but without the lid. For the time course experiments, a similar setup was used, but a chamber containing 4 ml of buffer was used, and successive sampling was performed.

Buffers—Krebs-Ringer phosphate buffer (used only for cell preparation and storage) had the following composition: 148 mM Na^+, 6.9 mM K^+, 1.5 mM Ca^{2+}, 1.4 mM Mg^{2+}, 119 mM Cl^-, 1.4 mM SO_4^{2-}, 5.6 mM $H_2PO_4^-$, 16.7 mM HPO_4^{2-}, 10 mM glucose, and 10 mM fructose. 4% crude bovine serum albumin was also included. The pH was adjusted with Tris-OH or Tris-HCl to 7.4.

Krebs-Ringer bicarbonate buffer (used for all experiments) had the following composition: 145 mM Na^+, 6.0 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 128 mM Cl^-, 1.2 mM SO_4^{2-}, 25.3 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, 10 mM glucose, 10 mM fructose, and 2% fatty acid-free bovine serum albumin. This buffer was purchased as a sterile solution from Statens Veterinär-

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medicinska Anstalt (Uppsala, Sweden). The buffer was bubbled with 5% CO_2 in air, and the pH was adjusted with Tris-OH or Tris-HCl to 7.4; the buffer was continuously bubbled with a small stream of 5% CO_2 in air until use.

Chemicals—Crude bovine serum albumin and fatty acid-free bovine serum albumin (albumin fraction V) were from Boehringer Mannheim. L-Norepinephrine bitartrate (Arterenol), forskolin, DL-propranolol, prazosin, yohimbine, A23187, ionomycin, chelerythrine, EGTA, and crude collagenase (type II; clostridiopeptidase A, EC 3.4.24.3) were obtained from Sigma (as the DL-form of propranolol was used, pA_2 values for L-propranolol are probably 0.3 units higher than the values given below). BRL 37344 was a gift from SmithKline Beecham Pharmaceuticals. Dobutamine (Dobutrex) was obtained as a solution for infusion from Lilly (Fegersheim, France). Salbutamol (Ventoline) was obtained as a solution for inhalation from Glaxo (Middlesex, UK). Cirazoline, KN-92, and KN-93 were obtained from Research Biochemicals International (Natick, MA). All adrenergic agents were freshly dissolved in water, except prazosin, which was dissolved in ethanol and diluted 1:10 in water for use. Forskolin and chelerythrine were dissolved and diluted in Me_2SO. A23187, KN-92, and KN-93 were dissolved in Me_2SO and diluted in water for use. EGTA was initially dissolved in dilute HCl.

Data Analysis—Dose-response curve data were, if not otherwise indicated, analyzed with the general fit option of the KaleidaGraph application for Macintosh for adherence to simple Michaelis-Menten kinetics, *i.e.* $V(x) = \text{basal} + V_{max}\cdot(x/(EC_{50} + x))$. The indicated uncertainties are those obtained from the fitting procedures. Where indicated, Michaelis-Menten kinetics with a free Hill coefficient were used for fitting, *i.e.* $V(x) = V_{max}\cdot(x^H/(EC_{50}{}^H + x^H))$, where H indicates the Hill coefficient.

When estimated from single-dose antagonist curve shifts, pA_2 values were calculated as $pA_2 = \log(C_R - 1) - \log[\text{antagonist}]$, where C_R is the ratio between the EC_{50} values in the presence and absence of antagonist. For multiple-dose antagonist curve shifts, Schild plots were used as illustrated.

RESULTS

cAMP Accumulation—To investigate the relationship between cAMP accumulation and thermogenesis in hamster brown fat cells, it was first necessary to establish the time course for adrenergically induced increases in cAMP levels.

In Fig. 1*A*, a time curve is shown for the norepinephrine-induced increase in cAMP content in suspensions of isolated brown fat cells. This experiment, as all following, was performed in the absence of any phosphodiesterase inhibitor; thus, it is the endogenously attained level of cAMP that is followed, rather than adenylyl cyclase activation as such, and the response includes the resultant hormonal effects, irrespective of whether they affect cAMP production or degradation.

As seen, the initial increase was rapid, and the elevated cAMP levels were maintained for at least 20 min. There was thus no tendency to the transient "overshoot" behavior earlier observed in brown fat cell preparations incubated under somewhat different conditions (16, 17), and the cAMP levels were at least as well maintained as the norepinephrine-induced thermogenesis (18).

Thermogenesis in these cells has been demonstrated to be mediated via β_3-receptors (15), and we therefore also investigated the cAMP response to a β_3-agonist, BRL 37344. This response had somewhat different kinetics than that to norepinephrine, and it did not reach the maximum before ≈10 min. At any time point, the level induced was slightly higher than that observed after norepinephrine. In all subsequent experiments, cAMP was determined 10 min after addition of agent.

Dose-response curves for the action of norepinephrine and BRL 37344 on cAMP levels are shown in Fig. 1*B*. High concentrations of norepinephrine were necessary to obtain a full effect on cAMP levels; the EC_{50} for norepinephrine for elevation of cAMP was ≈840 nM. This value should be contrasted with that associated with other effects of norepinephrine in these cells, such as thermogenesis (≤200 nM (*e.g.* Ref. 15 and see below).

In agreement with the results in the time course experiment, 1 μM BRL 37344 induced a higher level of cAMP than did 1 μM norepinephrine, although the maximal level attained by BRL 37344 was not different from that of norepinephrine. The EC_{50} was much lower than that for norepinephrine: 26 nM. Thus, as an elevator of cAMP levels in brown fat cells, BRL 37344 was 30-fold more potent than norepinephrine.

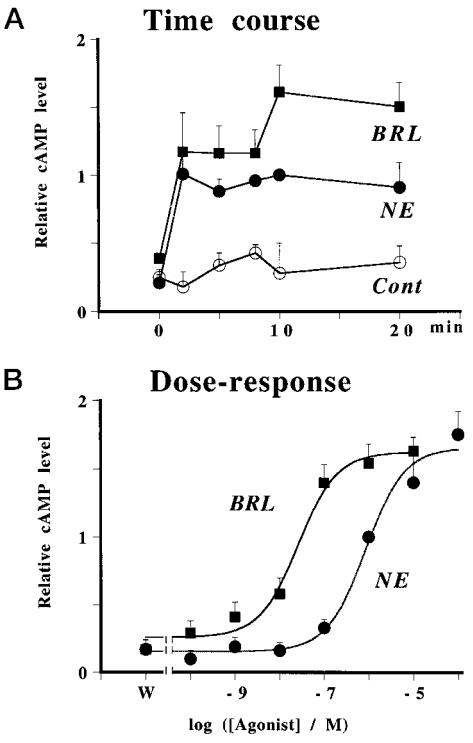

FIG. 1. **cAMP accumulation in isolated brown fat cells.** *A*, time course. The cells were incubated as described under "Experimental Procedures," with successive sampling from one chamber for each agent. At time 0, 1 μM BRL 37344, 1 μM norepinephrine (*NE*), or water (control (*Cont*)) was added, and samples were drawn at the indicated times. The points are means + S.E. from four independent cell preparations, normalized to norepinephrine at 10 min (the mean level was 34 ± 5 amol/cell). Here and in the following figures, the absence of S.E. indications signifies that they were smaller than the size of the symbol. *B*, dose-response curves for cAMP accumulation. Isolated brown adipocytes were incubated as described under "Experimental Procedures," with single incubations per point. The indicated concentrations of BRL 37344 or norepinephrine were added, and the incubations were terminated at 10 min after addition of agent. *W* indicates addition of solvent (water), followed by a 10-min incubation. The points are means + S.E. from five independent cell preparations, normalized to 1 μM norepinephrine. The mean level at 1 μM norepinephrine was 28 ± 3 amol/cell. Curves were drawn as detailed under "Experimental Procedures" for the best fit to simple Michaelis-Menten kinetics. The resulting values were, for norepinephrine, a basal value of 0.15 ± 0.04, a maximal increase of 1.50 ± 0.08, and an EC_{50} of 838 ± 219 nM ($r = 0.99$); for BRL 37344, the values were 0.26 ± 0.05, 1.36 ± 0.07, and 26 ± 7 nM, respectively ($r = 0.99$).

That the elevation of cAMP levels was more efficiently induced by BRL 37344 than by norepinephrine may in itself be seen as an indication that it is via stimulation of the β_3-adrenergic receptors that cAMP levels are increased. However, this does not unequivocally demonstrate that the stimulation occurs through β_3-receptors. Therefore, to establish through which β-receptor norepinephrine (and BRL 37344) elevates cAMP levels in brown fat cells, we utilized the characteristic of β_3-adrenergic receptors that they are relatively insensitive to conventional β-adrenergic antagonists, such as propranolol. For interaction of propranolol with β_1- or β_2-receptors, the pA_2 values should be 8–9, but with β_3-receptors, only ≈6 (19, 20). Therefore, dose-response curves for elevation of cAMP levels were constructed in the absence and presence of a fixed dose of propranolol. In Fig. 2*A*, it is shown that 8 μM propranolol was

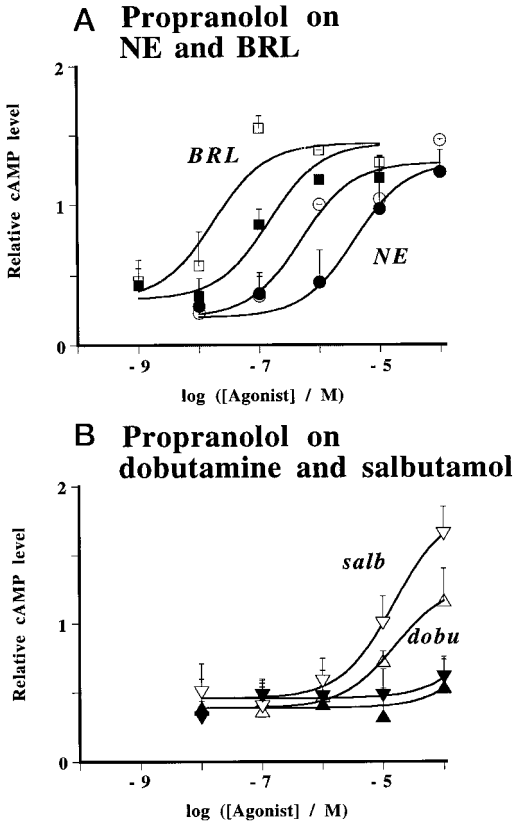

FIG. 2. **Effect of the β-adrenergic antagonist propranolol on the dose-response curves for cAMP formation for different agonists.** *A*, effect on BRL 37344 and norepinephrine (*NE*) dose-response curves. Dose-response experiments were carried out principally as described for Fig. 1*B*, with parallel incubations containing 8 μM propranolol (*closed symbols*) or not (*open symbols*). The points are means + S.E. from two independent cell preparations, normalized to 1 μM norepinephrine. Dose-response curves for the results in the absence of propranolol were drawn as detailed under "Experimental Procedures" (yielding basal, maximal, and EC_{50} values), and the data in the presence of propranolol were then evaluated with the same fitting procedure, but with the EC_{50} as the only free parameter. The apparent EC_{50} values for norepinephrine in the absence and presence of propranolol were 489 and 3759 nM, respectively, *i.e.* a C_R of 7.7 and a pA_2 for propranolol of 5.9. For BRL 37344, the values were 18 and 146 nM, respectively, with a C_R of 8.1 and a pA_2 for propranolol also of 5.9. *B*, effect on dobutamine (*dobu*) and salbutamol (*salb*) dose-response curves. Experiments and analysis were performed as described for *A*. The points are means + S.E. from three independent cell preparations. For dobutamine, the EC_{50} values in the absence and presence of propranolol were 16 and 542 μM, respectively, *i.e.* a C_R of 34 and a pA_2 for propranolol of 6.6. For salbutamol, the values were 15 and 810 μM, respectively, with a C_R of 53 and a pA_2 for propranolol of 6.8.

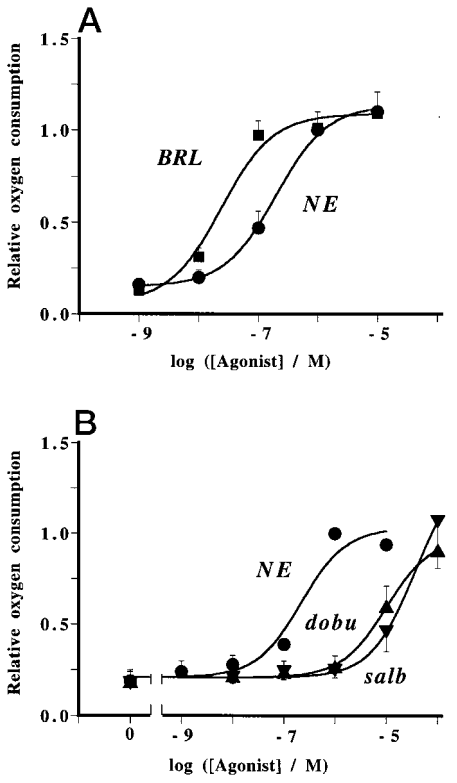

FIG. 3. **Dose-response curves for stimulation of oxygen consumption by β-adrenergic agonists.** Isolated brown adipocytes were incubated for determination of oxygen consumption as described under "Experimental Procedures." *A*, norepinephrine (*NE*) and BRL 37344. The indicated concentrations of the agents were added as a single addition (*cf.* Fig. 7, *first trace*), and the maximal respiration induced was then measured. The points are means + S.E. from four independent cell preparations; the results were normalized to the respiration obtained at 1 μM norepinephrine in each preparation (mean value of 667 ± 48 fmol O/min/cell). The EC_{50} for norepinephrine was 195 ± 26 nM, and that for BRL 37344 was 24 ± 11 nM. *B*, norepinephrine, dobutamine (*dobu*), and salbutamol (*salb*). The indicated concentrations of the agents were added as successive additions, and the stable level of respiration after each addition was measured. The points are means + or − S.E. from four independent cell preparations; the results were normalized to the respiration obtained at 1 μM norepinephrine in each preparation (mean value of 706 ± 97 fmol O/min/cell). The EC_{50} values were 219 ± 134 nM for norepinephrine, 9936 ± 1391 nM for dobutamine, and 36,858 ± 7594 nM for salbutamol.

sufficient to induce a significant shift of the dose-response curve for BRL 37344 to the right. The pA_2 for propranolol on BRL 37344-induced cAMP elevation was only 5.9, indicating that, as expected, BRL 37344 increased cAMP by interaction with β_3-receptors. When the effect of propranolol on norepinephrine was investigated, the pA_2 was also 5.9, implying that the physiological agonist norepinephrine also elevated cAMP levels through interaction with β_3-receptors.

Effect of Selective β_1- and β_2-Agonists—The above interpretation of the response to norepinephrine being mediated through β_3-receptors is based on the global response of the cells to norepinephrine (as if only one β-receptor type should be involved). It could not be excluded that a minor elevation was induced through other β-receptors. To increase the possibility of observing the activity of other β-adrenergic receptors, adrenergic agents that are selective for β_1- or β_2-receptors (here dobutamine and salbutamol) were tested.

In the dose-response curve for dobutamine (Fig. 2*B*), there was no measurable increase in cAMP at the low concentrations expected if dobutamine interacted with β_1-receptors. No significant elevation of cAMP content was observed at agonist concentrations below 10 μM, and the EC_{50} was as high as 16 μM. In the presence of 8 μM propranolol, only a marginal increase was observed at the highest dobutamine concentration, but this was still sufficient to estimate a pA_2 of 6.6. Thus, this selective β_1-agonist did not display a high potency for increasing cAMP levels (or even an observable component with a high potency), and the observable action was probably through interaction with the β_3-receptors. Thus, although β_1-receptors are found on these cells (21), we found no evidence for them being coupled to increased cAMP levels.

Very similar results were obtained with the selective β_2-agonist salbutamol (Fig. 2*B*). However, the absence of specific β_2-receptor interaction was less unexpected, as no evidence has been presented for the presence of β_2-receptors on these cells. It would therefore appear that in these isolated mature brown adipocytes, cAMP elevation is mediated only through β_3-adrenergic receptors.

Stimulation of Thermogenesis—All agents tested above in-

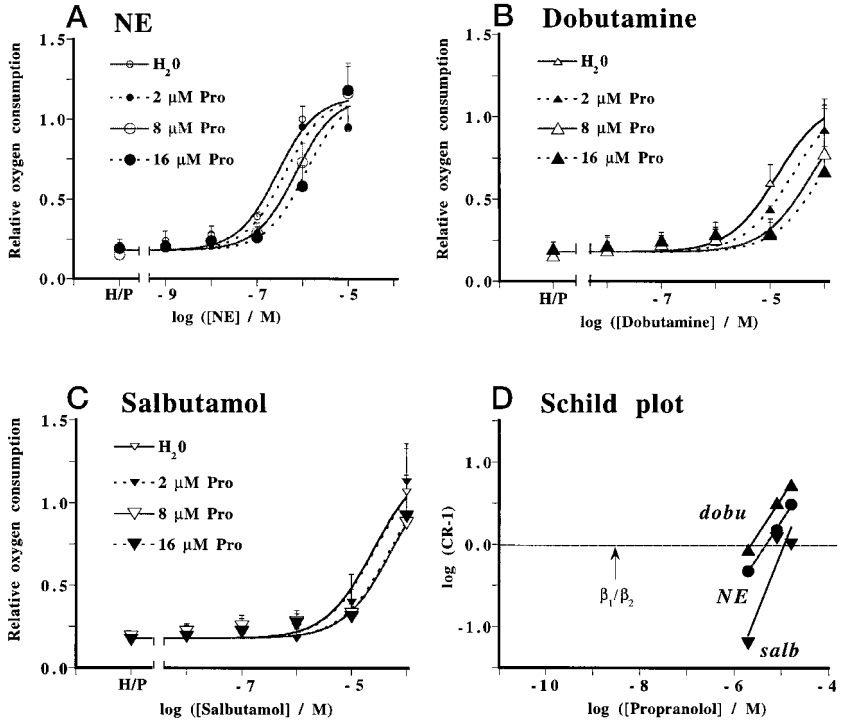

FIG. 4. **Effect of propranolol on dose-response curves for norepinephrine, salbutamol, and dobutamine stimulation of oxygen consumption.** *A–C*, experiments were performed principally as described for Fig. 3*B*, but in the presence of the indicated propranolol (*Pro*) concentrations. The points are means + S.E. from four independent cell preparations, with each agonist tested in each cell preparation. *H/P* indicates the rate after addition of water or propranolol. Data were analyzed as described for Fig. 2*A*, and the resulting C_R ratios were used for construction of *D*. *D*, shown is a Schild plot of propranolol inhibition of agonist-induced respiration. The lines are drawn based on the best linear fit. The resulting pA_2 values for propranolol were 5.6 against dobutamine (*dobu*; slope of 0.9), 5.3 against norepinephrine (*NE*; slope of 0.9), and 4.9 against salbutamol (*salb*; slope of 1.5). Also indicated is the expected pA_2 value for propranolol interaction with β_1- or β_2-receptors.

crease cAMP and are therefore predicted to stimulate thermogenesis. This was naturally the case for norepinephrine and BRL 37344 (Fig. 3*A*), as amply observed earlier. In the present investigation, the EC_{50} for norepinephrine was 195 nM, and that for BRL 37344 was 24 nM. Thus, for thermogenesis, there was the same qualitative difference between the agents as for cAMP elevation: BRL 37344 was more potent than norepinephrine. There was, however, a remarkable quantitative difference: although BRL 37344 was 30-fold more potent than norepinephrine as an elevator of cAMP levels, it was only 8-fold more potent as a stimulator of thermogenesis. This in itself implied that the relationship between cAMP elevation and stimulation of thermogenesis was not simple. The stimulation of thermogenesis by norepinephrine and BRL 37344 has earlier been shown to have a pA_2 for propranolol of $\approx$6 (15), indicating that both these agents are coupled through β_3-receptors (see also below).

It is to be expected that the increase in cAMP brought about by the selective β_1-agonist dobutamine or the selective β_2-agonist salbutamol would also lead to thermogenesis, but this has not earlier been demonstrated. In Fig. 3*B*, it is shown that these agents could also induce a full thermogenic response. To examine whether this response was mediated via a selective interaction of these agents with their intended receptors (β_1 or β_2) or unspecifically through β_3-receptors, dose-response curves for thermogenesis were obtained for these agents (and, for comparison, for norepinephrine) in the presence of three different propranolol concentrations. From these curves (Fig. 4, *A–C*), Schild plots were constructed, and apparent pA_2 values for propranolol were calculated (Fig. 4*D*). The pA_2 values were all between 5 and 6. Therefore, although the brown fat cells respond thermogenically to selective β_1- and β_2-agonists, it is clear that these agents mediated their effects through interaction with the β_3-receptors.

Relation between cAMP and Thermogenesis—Since cAMP production and oxygen consumption were thus demonstrated (above) to be β_3-receptor mediated processes, irrespective of which adrenergic agent was used, the prediction would be that, irrespective of which adrenergic agent is used, cAMP should be equally effective as a thermogenic stimulator.

In Fig. 5*A*, the rate of oxygen consumption (thermogenesis) stimulated by norepinephrine is plotted against the cAMP increase induced by norepinephrine; this is thus the dose-response curve for cAMP as a stimulator of thermogenesis. As expected, increases in cAMP levels correlated with increases in oxygen consumption until oxygen consumption peaked at the maximum capacity of the cells for thermogenesis. As can be understood from the graphs above and shown in Fig. 5*A*, higher norepinephrine concentrations could further increase the cAMP levels, but this was not associated with a further increase in thermogenesis.

Principally, a similar correlation was seen for the BRL 37344-stimulated increases in oxygen consumption and cAMP (Fig. 5*A*). However, there was an unexpected and important distinction: the relationships between cAMP levels and thermogenesis rates were not superimposable for the data sets for norepinephrine and BRL 37344. For any nonsaturating level of cAMP generated by BRL 37344 stimulation, the resulting thermogenesis was lower than that obtained with cAMP generated by norepinephrine stimulation. Only when cAMP levels were markedly increased by BRL 37344 was a full thermogenic response reached. Therefore, the view that there is a simple relationship between cAMP and thermogenesis is clearly not an adequate description of the situation.

A similar set of curves (Fig. 5*B*) was generated for dobutamine and salbutamol and compared with the norepinephrine response from the same series. It is again evident that the curves were not superimposable: cAMP generated by stimulation with the selective pharmacological ligands was apparently less thermogenically potent than that derived from norepinephrine stimulation. These differences in apparent cAMP potency are even more unanticipated when it is remembered that the increase in cAMP levels in all cases (both with norepinephrine and with the three selective adrenergic agents) is mediated through the same receptor, the β_3-receptor (as demonstrated above).

Apparent Thermogenic Potency of cAMP of Non-β-receptor Origin—The difference between the potency of cAMP to stim-

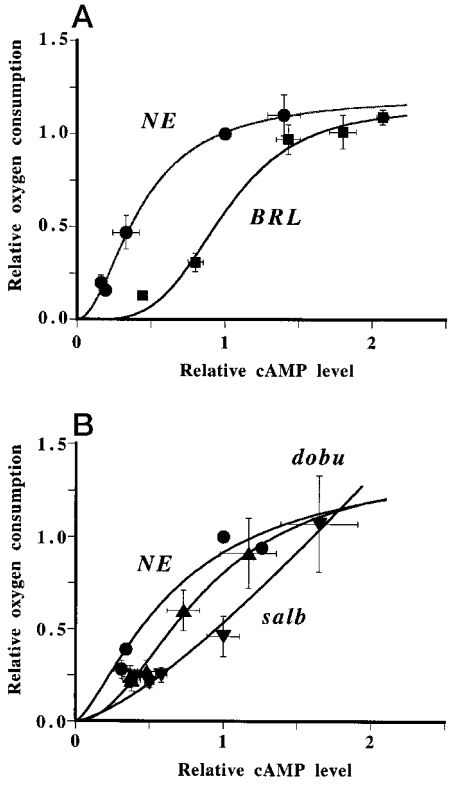

FIG. 5. **Correlation between induced cAMP levels and stimulation of oxygen consumption for different adrenergic agents.** *A*, correlation for norepinephrine (*NE*) and BRL 37344. The curves are based on the data in Figs. 1*B* and 3*A*. The curves were drawn as the best fit to Michaelis-Menten kinetics with a free Hill coefficient, as detailed under "Experimental Procedures." The apparent relative half-maximal value for cAMP as a stimulator of thermogenesis when the cells had been stimulated with norepinephrine was 0.43, and that when they had been stimulated with BRL 37344 was 0.99. *B*, correlation for norepinephrine, dobutamine, and salbutamol. The curves are based on the data in Figs. 2*B* and 3*B*. The curves were drawn as described for *A*. Here, the apparent relative half-maximal value with norepinephrine was 0.68, that with dobutamine (*dobu*) was 0.86, and that with salbutamol (*salb*) (read off the curve) was 1.2.

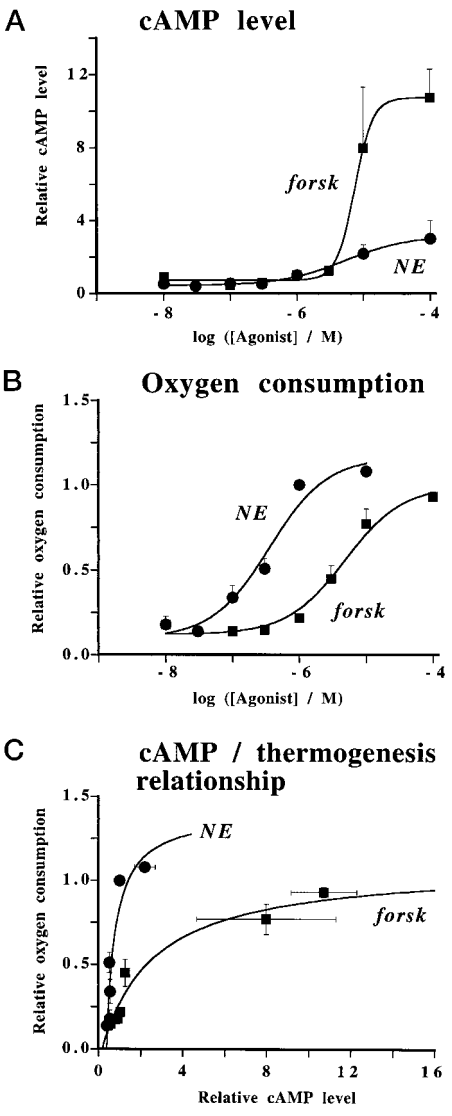

FIG. 6. **Dose-response curves for cAMP formation and thermogenesis induced by forskolin and norepinephrine.** *A*, cAMP determinations. Experiments were performed as described for Fig. 1*B*, with the indicated concentrations of agents. The points are means + S.E. from five independent cell preparations. The curves were drawn with a free Hill coefficient, as detailed under "Experimental Procedures." The EC_{50} for norepinephrine (*NE*) was 5223 ± 1237 nM, and that for forskolin (*forsk*) was 7364 ± 453 nM. *B*, oxygen consumption. Experiments were performed as described for Fig. 3*A*. The points are means + S.E. from six independent cell preparations. The curves were drawn with a Hill coefficient of 1. The EC_{50} for norepinephrine was 354 ± 150 nM, and that for forskolin was 4753 ± 1265 nM. *C*, correlation between induced cAMP levels and stimulation of oxygen consumption. The curves are based on the data in *A* and *B* and were drawn as described for Fig. 5*A*. The apparent relative half-maximal value for cAMP as a stimulator of thermogenesis when the cells had been stimulated with norepinephrine was 0.47, and that when they had been stimulated with forskolin was 2.98.

ulate thermogenesis when it originates from norepinephrine stimulation of the cells and when it originates from stimulation with the selective adrenergic agents could be due to a negative effect on thermogenesis of the selective agents or to a positive effect of norepinephrine as compared with the selective agents. To distinguish between these possibilities, it was necessary to increase cAMP levels in a non-adrenergic receptor-mediated way. Therefore, we investigated the thermogenic potency of cAMP generated by stimulation with forskolin, which directly stimulates adenylyl cyclase.

In Fig. 6*A*, dose-response curves for the action of forskolin on the level of cAMP, compared with norepinephrine, are presented. It is evident (and in agreement with Ref. 22) that forskolin was able to massively stimulate cAMP production, far in excess of the levels generated by norepinephrine. However, when the effect of forskolin as a stimulator of thermogenesis was investigated (Fig. 6*B*), a remarkable reversal was observed: although forskolin was able to stimulate thermogenesis, norepinephrine was more potent. Forskolin was not even able to fully reach the level of norepinephrine-induced thermogenesis.

Curves were therefore again constructed to analyze the relationship between cAMP levels and oxygen consumption. As is clear from Fig. 6*C*, a similar, but even more dramatic difference was seen between norepinephrine and forskolin as between norepinephrine and the selective agents (Fig. 5). The thermogenic potency of forskolin-generated cAMP was apparently markedly lower than that generated by norepinephrine. Thus, the higher thermogenic potency of cAMP generated by norepinephrine stimulation compared with that generated by the selective adrenergic agents (Fig. 5) was likely due to an additional positive effect of norepinephrine.

An α_1-Adrenergic Potentiating Effect—There is, of course, an important difference between norepinephrine and the other agents studied here: norepinephrine interacts with adrenergic receptor subtypes other than β, *i.e.* α_2- and α_1-adrenoreceptors. Concerning possible α_2-adrenergic effects, it has earlier been

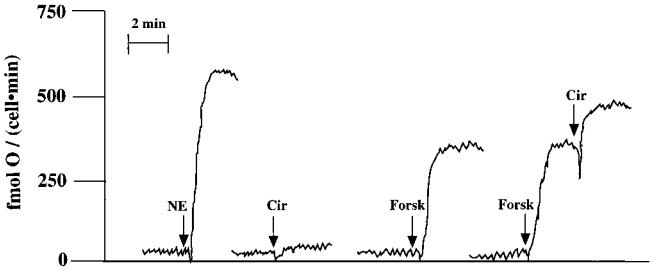

FIG. 7. **Interaction between forskolin and cirazoline in stimulation of oxygen consumption in isolated brown adipocytes.** Cell suspensions were incubated and rates of oxygen consumption were determined as described under "Experimental Procedures." Shown is a representative experiment from one cell preparation. Additions were 1 μM norepinephrine (*NE*), 1 μM cirazoline (*Cir*), and 3 μM forskolin (*Forsk*).

demonstrated that hamster brown fat cells apparently lack α_2-adrenergic receptors (23), and this pathway is therefore probably not relevant.

However, isolated brown fat cells have a high density of α_1-adrenergic receptors (9) coupled to activation of phosphatidylinositol bisphosphate turnover (24), protein kinase C activation (25), inositol 1,4,5-trisphosphate production (26), and an increase in cytosolic Ca^{2+} (27). In the present circumstances, the possibility therefore existed that it could be through interaction with α_1-receptors that norepinephrine potentiated the thermogenic effect of the β-adrenergically generated cAMP. Studies were therefore performed to investigate whether selective α_1-adrenergic stimulation could potentiate the thermogenic response to a given cAMP level. To avoid possible problems with receptor specificity for selective adrenergic agents, we performed these studies in cells in which the cAMP level was elevated with forskolin. A dose of forskolin was chosen that (according to the data in Fig. 6*A*) gave an increase in cAMP approximately similar to that induced by the dose of norepinephrine that gave maximal oxygen consumption.

First, we investigated the effect of α_1-adrenergic stimulation on thermogenesis. Results from a typical experiment are seen in Fig. 7. Forskolin, at the dose utilized, stimulated oxygen consumption to some 60% of the level stimulated maximally by norepinephrine. Cirazoline, a selective α_1-adrenergic receptor agonist, was in itself unable to stimulate oxygen consumption at the concentration utilized here (and indeed at any concentration between 1 nM and 10 μM) (data not shown here, but compare with Fig. 9*A*). However, when the cells were stimulated with cirazoline in combination with forskolin, the forskolin-stimulated respiration markedly increased, irrespective of whether cirazoline was added together with or (as shown here) after forskolin.

In Fig. 8*A*, a dose-response curve is shown for this cirazoline-mediated potentiation of forskolin-induced thermogenesis. The response was dose-dependent, with an EC_{50} of 23 nM, *i.e.* within the order of affinity expected for a cirazoline effect on α_1-receptors. To confirm that the cirazoline-induced potentiation was indeed mediated via α_1-adrenergic receptors, the effects of antagonists to α_1- and α_2-receptors on the potentiation were studied (Fig. 8*B*). The selective α_2-antagonist yohimbine was without significant effect, whereas the selective α_1-antagonist prazosin completely blocked the cirazoline-mediated effect. Thus, provided that an increase in cAMP had been induced by forskolin, α_1-adrenergic stimulation was able to nearly double the thermogenic effect of this amount of cAMP.

To eliminate the possibility that the increase in thermogenesis caused by α_1-adrenergic stimulation was due to an unexpected effect of cirazoline on cAMP accumulation, cAMP levels and thermogenesis were determined in parallel in the presence

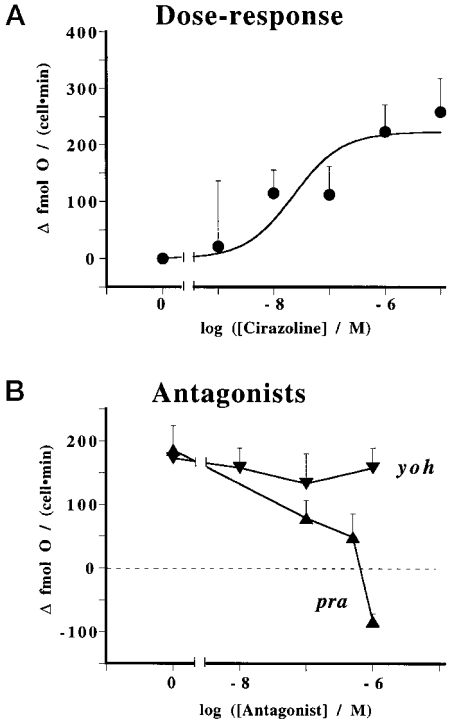

FIG. 8. **Cirazoline potentiation of forskolin-induced thermogenesis.** *A*, dose-response curve for cirazoline-induced potentiation. Experiments were performed principally as described for Fig. 7 (*fourth trace*), but forskolin and cirazoline were added simultaneously. The cirazoline-induced increase (Δ oxygen consumption) was determined as the oxygen consumption rate in traces obtained with different cirazoline concentrations minus the rate when only forskolin was added. The points are means + S.E. from four experiments on the same number of independent cell preparations. The EC_{50} for cirazoline was 23 $\pm$ 20 nM. *B*, influence of α-adrenergic antagonists on cirazoline-induced potentiation. Experiments were performed principally as described for Fig. 7 (*fourth trace*), except that different concentrations of prazosin (*pra*) or yohimbine (*yoh*) (or ethanol/water vehicle) were added 2.5 min prior to addition of forskolin, and cirazoline and forskolin were added simultaneously. The calculations were done as described for *A*. The points are means $\pm$ S.E. from three independent cell preparations.

of forskolin and with varying cirazoline concentrations. The relationship between cAMP levels and oxygen consumption is plotted in Fig. 9*A*.

It is shown (Fig. 9*A*, *open symbols*, *lower left corner*) that cirazoline in itself increased neither thermogenesis nor cAMP levels. The concentration of forskolin used here (*point F*) gave a level of cAMP very close to that obtained with 1 μM norepinephrine (*point NE*), but only half the thermogenesis. The further presence of cirazoline (*closed diamonds*) had no dose-dependent effect on cAMP levels, but was maximally able to approximately double thermogenesis. Thus, although unable in itself to stimulate oxygen consumption and without any innate ability to elevate cAMP levels, cirazoline dramatically potentiated the forskolin-stimulated respiration.

Mediation of the α_1-Adrenergic Effect—The intracellular signal transduction of this α_1-adrenergic potentiation of the forskolin-stimulated respiration could be through either of the two arms of the α_1-receptor pathway, *i.e.* either via an activation of protein kinase C or via an increase in $[Ca^{2+}]_i$.

To investigate whether the effect was protein kinase C-mediated, the cells were treated with various concentrations of the protein kinase C inhibitor chelerythrine prior to stimulation with forskolin plus cirazoline. As is evident in Table I, chelerythrine had no effect on the (forskolin + cirazoline)-stimulated respiration. It would thus appear that protein kinase C was not involved in the signal transduction of this effect of

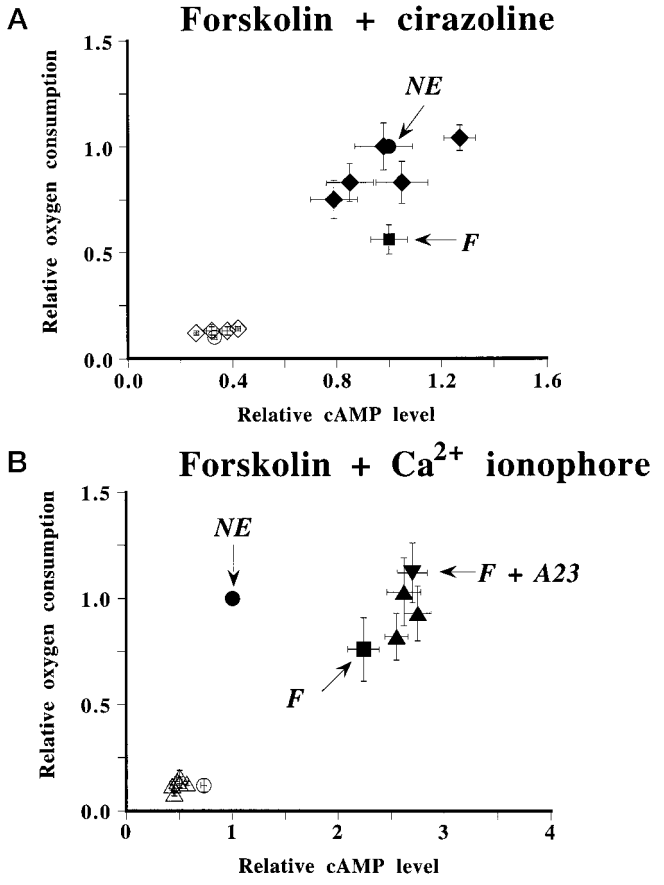

FIG. 9. **Effect of cirazoline and calcium on the correlation between induced cAMP levels and stimulation of oxygen consumption.** *A*, effect of cirazoline. Experiments were performed as described for Fig. 6. In each experimental series, the response to 1 μM norepinephrine (*NE*) was determined and used to normalize the results. The results of addition of different concentrations of cirazoline (1, 10, 100 nM, 1 and 10 μM) are plotted as *open symbols*. The effect of 3 μM forskolin (*F*) alone is indicated, and the results of adding forskolin plus the different cirazoline concentrations are shown (*closed diamonds*; from *left* to *right*, the *diamonds* represent the following cirazoline concentrations: 1 nM, 100 nM, 10 nM and 10 μM). The points are means ± S.E. from four independent cell preparations in which oxygen consumption and cAMP levels were determined in parallel. *B*, effect of Ca^{2+} ionophores. Experiments were performed as described for *A*, except that either ionomycin at different concentrations (*closed triangles*; from the lowest point: 0.1, 1, and 10 μM) was used instead of cirazoline or 10 μM A23187 (*F + A23*; *inverted closed triangle*) was used. The points are means ± S.E. from four independent cell preparations in which oxygen consumption and cAMP levels were determined in parallel.

TABLE I
Influence of the protein kinase C inhibitor chelerythrine on oxygen consumption stimulated by forskolin plus cirazoline

The experiments were performed as exemplified in Fig. 7 (*last trace*), except that forskolin and cirazoline were added simultaneously and the indicated additions of chelerythrine were made 3 min before addition of forskolin + cirazoline. Values are means ± S.E. from three experiments, performed on independent cell preparations.

	Basal	+Forskolin
H_2O	98 ± 17	435 ± 51
Cirazoline	105 ± 35	585 ± 26
+0.1 μM chelerythrine	99 ± 21	593 ± 61
+1 μM chelerythrine	102 ± 13	586 ± 43
+10 μM chelerythrine	86 ± 18	643 ± 8

cirazoline.

It was therefore likely that the cirazoline effect was mediated by an increase in cytosolic calcium levels. If this was the case, the effect of cirazoline should be mimicked by Ca^{2+} ionophores. Therefore, an experiment similar to that in Fig. 9*A* was performed, but various concentrations of the calcium ionophore ionomycin were used instead of cirazoline. The results are shown in Fig. 9*B*. Ionomycin in itself affected neither cAMP levels nor thermogenesis (*open symbols*, *lower left corner*). However, when ionomycin was added to cells together with forskolin, it was again possible to dose-dependently increase thermogenesis from the forskolin level (*point F*) to the level obtained with norepinephrine (*point NE*) without any systematic effect on cAMP levels. A similar effect was seen with the Ca^{2+} ionophore A23187 (*point F + A23*). Both Ca^{2+} ionophores could thus mimic the effect of α_1-adrenergic stimulation, indicating that an elevation of intracellular calcium was a sufficient signal to potentiate forskolin-induced respiration in a cAMP-independent manner.

The calcium causing the potentiation could originate from intracellular stores or from an increased influx from the medium. We have earlier found that even in the absence of extracellular Ca^{2+}, α_1-adrenergic stimulation can increase $[Ca^{2+}]_i$ in these cells (27), although to a somewhat lower level than in its presence. To investigate the origin of the Ca^{2+} required for the potentiating effect, we chelated extracellular Ca^{2+} with EGTA prior to addition of forskolin plus cirazoline. We observed no significant effect of EGTA on the magnitude of the cirazoline potentiation of thermogenesis (158 ± 11 fmol O/cell/min in the absence of EGTA and 141 ± 33 in the presence of 5 mM EGTA; means ± S.E. from four independent experiments). It would thus appear that the calcium for the potentiating effect could be derived from intracellular stores.

The effects of the α_1-receptor-induced increase in cytosolic calcium levels may be mediated through activation of calmodulin-dependent protein kinase. To evaluate if this kinase was a necessary step in the calcium-mediated cirazoline effect, a 1 μM concentration of the calmodulin-dependent protein kinase inhibitor KN-93 or the inactive analogue KN-92 was added to the cells prior to forskolin + cirazoline. Neither agent influenced the magnitude of the cirazoline potentiation of thermogenesis (data not shown). Thus, calmodulin-dependent protein kinase is probably not the route by which the calcium activation of respiration is achieved.

The potentiation of thermogenesis caused by α_1-adrenergic stimulation could be localized to different steps between the increased cAMP levels and the final thermogenic result. It could be possible that α_1-adrenergic stimulation in itself had an uncoupling effect, allowing for thermogenesis provided that substrate was otherwise available. The substrate would physiologically be provided by β-receptor-stimulated lipolysis, but it is possible experimentally to provide substrate by addition of pyruvate. Pyruvate (at a concentration of 5 mM) had virtually no effect on thermogenesis, but when an artificial uncoupler (FCCP,[1] 100 μM) was added, a clear thermogenic response (~45% of that to norepinephrine) was induced; such a response was not observed if no pyruvate was present. If α_1-adrenergic stimulation in itself was able to uncouple, a response to cirazoline (in the presence of pyruvate) similar to that to FCCP would be expected, but cirazoline was practically without effect under these circumstances (data not shown). Thus, α_1-adrenergic stimulation probably had no uncoupling effect in itself.

A further possibility would be that α_1-adrenergic stimulation would provide extra substrate for thermogenesis (in addition to that provided by lipolysis). Thus, when the mitochondria became uncoupled due to β-adrenergic stimulation, both the substrate from lipolysis and that emanating from α_1-adrenergic

[1] The abbreviation used is: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

stimulation would be combusted. We therefore tested whether the thermogenic response to the uncoupler FCCP would be larger if the cells were first stimulated with cirazoline; this was, however, not the case (data not shown). Thus, α_1-adrenergic stimulation probably did not provide additional thermogenic substrate.

It could also be suggested that α_1-adrenergic stimulation, *e.g.* through its effects on cytosolic Ca^{2+} levels, could facilitate the combustion of thermogenic substrate from β-receptor-stimulated lipolysis. This could occur through Ca^{2+}-induced stimulation of enzymes of the citric acid cycle (28) or through mitochondrial matrix expansion caused, for example, by activation of the K^+ channel (29); such matrix expansion would facilitate thermogenesis (30). In an attempt to investigate this, we examined if cirazoline would augment FCCP-induced thermogenesis with pyruvate as substrate. However, this was not the case (data not shown). Thus, at least under these experimental conditions, α_1-adrenergic stimulation did not facilitate substrate combustion, but the situation may, of course, be different under physiological conditions when flow through the citric acid cycle is greater than that occurring with pyruvate as added substrate.

In studies parallel to those above, we examined if raising cytosolic Ca^{2+} levels by the ionophore A23187 (100 nM to 10 μM) would induce the features discussed above. The results with this Ca^{2+} ionophore were practically identical to those obtained with cirazoline; thus, neither α_1-adrenergic stimulation nor an increase in $[Ca^{2+}]_i$ was able to uncouple the mitochondria, to provide additional substrate, or to facilitate substrate combustion under the conditions tested. Thus, the potentiating effect of α_1-adrenergic stimulation on the ability of cAMP to induce thermogenesis is apparently located at a step between increased cAMP levels and physiological uncoupling of the brown fat mitochondria.

DISCUSSION

In this investigation, we first demonstrated that the adrenergically mediated increase in cAMP levels in hamster brown fat cells is mediated via β_3-receptors, irrespective of which adrenergic agent is used. Despite this homogeneous mediation of cAMP formation, we observed that cAMP derived from norepinephrine stimulation was apparently more thermogenically potent than cAMP generated from stimulation with selective adrenergic agonists or with forskolin. We demonstrated that this difference was due to the α_1-adrenergic component of norepinephrine stimulation, which was without thermogenic effect itself but which augmented the apparent thermogenic effect of cAMP. This α_1-adrenergic component was mediated via an increase in $[Ca^{2+}]_i$. The α_1-adrenergic effect was not directly on the mitochondrial thermogenic process. The observations confer to the α_1-receptor pathway a quantitatively significant role in the acute regulation of thermogenesis.

Only β_3-Receptors Are Involved in cAMP Elevation in Hamster Brown Fat Cells—Our results ascribe the adrenergically mediated increase in cAMP levels solely to stimulation of β_3-receptors and give no indication of a β_1-receptor involvement in this process. This was seen both by the high sensitivity of cAMP formation to activation by the selective β_3-agonist BRL 37344 and by the low sensitivity to activation by the selective β_1-agonist dobutamine and the selective β_2-agonist salbutamol. It was also clear from the interaction of the β-antagonist propranolol with these agents that the pA_2 values obtained in all four cases clearly indicated interaction with β_3-receptors, and the monophasic curves obtained did not indicate interaction with multiple receptors.

The results obtained here for brown fat cells from Syrian hamsters (that only β_3-receptors are involved in the adrenergic elevation of cAMP levels) may not necessarily be valid for brown fat cells from all species. The involvement of the different β-receptor subtypes in stimulation of adipose tissues is apparently very species-specific, and also the pharmacology of the β_3-receptor is apparently variable between species (7, 31). The clear-cut result obtained for the hamster cells facilitates, however, interpretation of the relationship between cAMP elevation and thermogenesis stimulation (below).

This result implies that the β_1-receptors, earlier characterized on these isolated hamster brown fat cells (21), are not coupled to classical mediation through cAMP, and they appear therefore not to be functional. This may be due to a decoupling occurring during cellular differentiation; at least in mouse and rat brown preadipocytes in culture, the β_1-receptors are coupled (32–34) and stimulate cell proliferation (32, 35, 36) (although the predominant receptor after differentiation also in these cells is the β_3-receptor (32)).

Relationship between Cellular cAMP Levels and Thermogenesis—Since the formation of cAMP by all adrenergic agonists occurred through the same receptor, it would be expected that the resulting level of cAMP would correlate well with the stimulation of thermogenesis. It was, however, evident that cAMP generated by norepinephrine was more efficient in stimulating thermogenesis than that generated by other β-adrenergic agonists or by forskolin. We analyzed this difference in apparent potency and demonstrated that it was due to the simultaneous activation of α_1-adrenergic receptors by norepinephrine.

Another formulation of the difference in apparent thermogenic potency of cAMP derived from norepinephrine stimulation and that derived from, for example, forskolin stimulation could be that the cAMP in these cells is functionally compartmentalized: some forskolin-derived cAMP could be postulated to be in a compartment not coupled to thermogenesis. In this formulation, α_1-adrenergic stimulation may be said to direct the cAMP to a better coupled functional compartment.

A compartmentalization of cAMP has been discussed in other systems (*e.g.* Ref. 37), often under conditions where, for example, forskolin-derived cAMP and cAMP derived from hormonal stimulation apparently do not induce the final cellular response with the same potency, *i.e.* a situation akin to the one described here for brown fat cells. Based on the results presented here, it may be suggested that in some of these cases, a parallel hormonal stimulation of, for example, an inositol 1,4,5-trisphosphate/$[Ca^{2+}]_i$ pathway may also convey to the cAMP a full effector capacity.

Why Has This Effect of α_1-Adrenergic Stimulation Been Overlooked?—It may seem surprising that such a quantitatively significant effect of α_1-adrenergic stimulation has previously gone unnoticed in brown fat cells. However, direct comparisons between cAMP levels and thermogenesis (as those shown here in Figs. 5 and 6*C*) have only rarely been presented and only with norepinephrine as the stimulatory agent (*e.g.* Ref. 38); thus, no comparison between the relative efficiencies of different agents has been made. Furthermore, the ability of cAMP-elevating agents to stimulate thermogenesis to practically the same level as that observed with norepinephrine has been taken as an indication that only cAMP is involved in the signaling process. In reality, these experiments only demonstrated that (high) cAMP *can* elicit full thermogenesis, not that this is what actually occurs during physiological stimulation. Also in the interpretation of the effect of norepinephrine, it is clear that the exact experimental conditions affect the interpretation. Thus, at a concentration of norepinephrine that is supersaturating for thermogenesis, a sufficiently high level of cAMP would be formed to fully induce thermogenesis, even if the α_1-adrenergic component observed here were experimen-

tally inhibited. Thus, the erroneous conclusion would be that no α_1-adrenergic component is involved in the adrenergic effect. However, as seen here, at all concentrations of norepinephrine at which the cells demonstrate a graded response to the agonist, the α_1-adrenergic component is apparently of quantitatively similar significance to the β-adrenergic component, and this is probably the physiologically most relevant condition.

There is also an interesting observation extending the significance of the present results. Noronha *et al.* (39) found that in brown fat cells isolated from normal rats, no evidence for an α_1-adrenergic component in thermogenesis could be discerned. However, in brown fat cells isolated from hypothyroid rats, it was possible to observe an enhanced thermogenesis by α_1-adrenergic stimulation. Based on the present experiments, it may be postulated that in the cells from normal rats, forskolin was able to increase cAMP to the unphysiologically high level where it can alone elicit practically full thermogenesis (*cf.* Fig. 6). However, in cells from hypothyroid rats, forskolin had a diminished ability to elevate cAMP levels (40), and only "physiological" cAMP levels were attained. Under these circumstances, as demonstrated here, an α_1-adrenergic stimulation could confer full thermogenic potency to the cAMP, and therefore, an α_1-adrenergic effect became observable in this pathological state, even when forskolin was used to stimulate adenylyl cyclase.

In this study and in Ref. 39, α_1-adrenergic stimulation in itself had no ability to stimulate thermogenesis; the effect was only synergistic. This is apparently in contrast to earlier observations that selective α_1-receptor activation in itself stimulated a small amount of oxygen consumption (10–20% of maximal norepinephrine) (12, 13, 41). However, considering the marked ability of α_1-adrenergic stimulation demonstrated here to increase the thermogenic potency of small amounts of cAMP, it may be suggested that it was such a potentiating effect that was seen, as the actual conditions then used may have led to stimulation of cAMP formation, the thermogenic effect of which was then augmented by the α_1-adrenergic stimulation.

An Increasing Understanding of the Significance of α_1-Adrenergic Receptors in Brown Adipose Tissue—α_1-Adrenergic receptors were first characterized in brown fat cells by radioligand binding studies with prazosin (9). Many features of these receptors have subsequently been described, including up- and down-regulation in relation to sympathetic stimulation (10, 42–45) and coupling of α_1-receptor stimulation to activation of second messengers and ionic events in brown fat cells (22, 24, 27, 46–52). However, the physiological role of the α_1-receptors has remained unclear, and reported physiological effects of selective α_1-adrenergic stimulation have been minor, especially when compared with the fact that the levels of α_1-receptor mRNA in the tissue are among the highest in the body (45).

This study may be seen as one in an emerging series in which evidence is presented indicating that the importance of α_1-adrenergic stimulation is its positive and often synergistic interaction with β-adrenergic responses. For regulation of the expression of the uncoupling protein UCP1 (53, 54) and lipoprotein lipase (55), an α_1-adrenergic component has been identified, and a synergistic interaction between α_1- and β-receptors and signaling pathways has been demonstrated for both thyroxine deiodinase (40) and c-*fos* (22) expression. Here, unexpectedly, we demonstrate that even for a process that has been accepted to be essentially β-adrenergically stimulated and cAMP-mediated, a strong α_1/β-receptor synergism can be observed.

Physiological stimulation with norepinephrine has broad effects on brown adipose tissue (56). Many important processes in these cells have been described to be positively controlled by cAMP, including mitochondriogenesis in general (57), differentiation in general (58), and, somewhat unusually, even brown fat cell proliferation (32, 36). However, in few of these cases has a thorough analysis of the significance of cAMP been made, and in the light of the present observations, where an α_1/β-receptor synergism is very apparent in a process earlier believed to be essentially cAMP-mediated, it may be suggested that some of the functions earlier claimed to be β-receptor/cAMP-mediated may be reanalyzed to unveil significant α_1-adrenergic effects. Thus, the present demonstration of the α_1/β-receptor and $[Ca^{2+}]_i$ cAMP synergism in the acute thermogenic function of the tissue may also be of significance for the understanding of the recruitment process that augments the total thermogenic capacity of the tissue.

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