A Dual Component Analysis Explains the Distinctive Kinetics of cAMP Accumulation in Brown Adipocytes*

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The mechanism behind the distinctive non-Michaelis-Menten, bell-shaped kinetics of cAMP accumulation in brown adipocytes (which underlies the similar kinetics of UCP1 and β3-adrenergoreceptor gene expression) was investigated. A theoretical dual component analysis indicated that the observed dose-response curves could be constructed as the resultant of a stimulatory and an inhibitory component. Experimentally, inhibition of the α-component of the norepinephrine response revealed the underlying existence of a much larger stimulatory β-component which displayed monophasic Michaelis-Menten kinetics. The inhibitory α-component (which was also monophasic but had a 2-fold higher EC50) was mediated via an increase in [Ca2+]i; the protein kinase C pathway was not involved. The [Ca2+]i increase which resulted in massive inhibition of cAMP accumulation was very low: <100 nM. The [Ca2+]i signal stimulated a calmodulin-controlled phosphodiesterase, possibly PDE-1. The requirement of this specific interaction pattern between β- and α-adrenergic stimulation was thus part of the differentiation program of the brown adipocytes. It was concluded that an array of synergistic or inhibitory α/β interactions occur in the adrenergic regulation of this cell type which is unique in its dependence upon adrenergic stimulation for cellular proliferation, differentiation, and metabolic function.

When stimulated with norepinephrine, brown adipocytes express the gene for the uncoupling protein-1 (UCP1)1 (1–4). However, the kinetics of the induction of the expression of this gene do not adhere to simple, monophasic Michaelis-Menten kinetics. Rather, a distinctive bell-shaped response is observed, gene do not adhere to simple, monophasic Michaelis-Menten kinetics. However, the kinetics of the induction of the expression of this pathway was not involved. The [Ca2+]i concentration being less efficient (3, 4).

The observation of non-Michaelis-Menten kinetics such as these may easily be considered an experimental artifact and/or interpreted as an indication of "overstimulation" of the relevant receptor, leading to an acute "desensitization" of the response. Because of difficulties in determining kinetic parameters in non-Michaelis-Menten-responding systems, the downward part of such dose-response curves may often be ignored (or perhaps even omitted) in the presentation of data.

However, in a theoretical analysis, Rovati and Nicosia (5) demonstrated that one conceivable possibility for generation of bell-shaped dose-response curves would be that these curves represent the resultant of interacting stimulatory and inhibitory components. They also implied that in such systems, large underlying stimulatory components, masked by the inhibitory component, could be revealed.

Following this proposal, we have here attempted to resolve the bell-shaped dose-response curve for norepinephrine into dual components. As the kinetics of UCP1 gene expression have been demonstrated to mirror those of norepinephrine-induced cAMP accumulation in these cells (6), we have, de facto, examined whether specific receptors and intracellular mediators could interact to create the unusual kinetics of cAMP accumulation.

We conclude that a Rovati/Nicosia model fully explains the kinetics of the control of cAMP levels in brown adipocytes and that through the elimination of the inherent inhibitory component, a strikingly high but otherwise masked potential for stimulation of cAMP accumulation via β3-receptors may be revealed.

EXPERIMENTAL PROCEDURES

Cell Isolation—Brown fat precursor cells were isolated from 3–4-week-old male mice of the NMRI strain, principally as described by Nèchad et al. (7). Tissue was combined from the cervical, interscapular, and axillary depots and incubated in a Hepes-buffered solution (7), containing 200 units/ml crude collagenase type 2 (Sigma).

Cell Culture—Cells were routinely cultivated in 12-well plates (growth area 3.83 cm²/well) as described earlier (3, 6, 8) in 1 ml of a culture medium consisting of Dulbecco’s modified Eagle’s medium (Flow) 1 × liquid without glutamine, 4 μM glutamine (Flow) added supplemented with 10% newborn calf serum (Flow), 4 μg insulin (Atrapid Human, Novo), 10 μM Heps (Flow), and 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate (Kebo) per ml, at 37 °C in a water-saturated atmosphere of 8% CO2 in air in a Heraeus Rapid Human, Novo), 10 m M Hepes (Flow), and 50 IU of penicillin, 50 μg of streptomycin, and 25 μg of sodium ascorbate (Kebo) per ml, at 37 °C in a water-saturated atmosphere of 8% CO2 in air in a Heraeus CO2-auto-zero B5061 incubator. The medium was completely exchanged with fresh prewarmed medium on day 1 (when the kinetics were first washed with 2 ml of prewarmed Dulbecco’s modified Eagle’s medium) and on days 3 and 6 (without wash).

cAMP Determinations—On day 6 or 7, as indicated, antagonists, PMA, or phosphodiesterase inhibitors were added 5–7 min, and cirazoline or isononycin 1 min, before (other) agonist addition. After a further 20–25 min, the culture medium was aspirated, 0.8 ml of 95% ethanol added, the cells scraped off, and the suspension transferred to Eppendorf tubes. The wells were washed with 0.5 ml of 70% ethanol, and the combined suspensions were dried in a Speedvac centrifuge. The dried samples were dissolved in 0.3–1 ml of the Buffer 1 provided with the Cyclic AMP [3H]Assay System from Amersham Pharmacia Biotech and centrifuged at 14,000 rpm, 10 min. Two 50-μl aliquots of the supernatants of every sample were analyzed according to the description in the assay system. For every concentration of any agent in each experiment,
two wells were used. Thus, in each experiment, each value used in later calculations is the mean of the four measurements of cAMP. The results were routinely normalized in each series of experiments by setting the value of the cAMP response for 0.1 μM NE (or 0.1 μM isoprenaline or 1 μM CGP-12177) to 100%.

**Measurement of Intracellular Ca²⁺** —The concentration of cytosolic Ca²⁺ was measured with the Fura-2 fluorescent dye technique (9), principally as earlier described (10), in a dual wavelength spectrophotometer (Sigma ZWS II) with alternating excitation wavelengths of 355 and 395 nm, with an emission cut-off filter (KV 470). The ratio of emission was sampled with frequency of 2.5/s and smoothed by a running means technique (15 samples). Intracellular Ca²⁺ concentrations ([Ca²⁺]i) were calculated and graphs drawn (KaleidaGraph) from these data using the Grynkiewicz et al. (9) equation: 

\[ [Ca^{2+}] = K_0 \frac{R - R_{\text{max}}}{[R - R_{\text{max}}] - R/F_i - F/_{\text{max}}} \]

where \( R \) is the running 355/395 fluorescence ratio, \( R_{\text{max}} \) is this ratio under Ca²⁺-free conditions, \( F_i \) is the actual fluorescence at 395 nm under Ca²⁺-free conditions, and \( F_{\text{max}} \) and \( R_{\text{max}} \) are these values under saturating Ca²⁺ conditions. The \( K_0 \) value used was that determined by Grynkiewicz et al. (9), i.e. 224 nm. Experimentally, each experimental trace was finalized by adding to the cuvette 30 μM ionomycin (yielding the \( R_{\text{max}} \) and the \( F_i \) values), followed by an addition of 20 μM digitonin with 2 mM MnCl₂, yielding the \( R_{\text{max}} \) and the \( F_i \) values. Under the conditions used here, the \( F_i/F_{\text{max}} \) value was very close to 1.

**Analysis of Dose-Response Curves** —For analysis of dose-response curves, the non-linear regression analysis curve-fitting option of the KaleidaGraph 3.0 application was used. Monophasic dose-response data were analyzed with the rearranged Michaelis-Menten equation,

\[ V_a = \frac{V_{\text{max}}(S) + V_{\text{max}}(A)}{1 + (IC_{50}(A)^3)} \]  

(1)

where \( h \) is the Hill coefficient. If \( h \) was estimated to be close to 1 in the initial analysis, the data were recalculated with \( h = 1 \). For the analysis of the biphasic ("semi-bell-shaped") dose-response data, a model (5) for the interaction of a ligand with two different receptors, one stimulatory (S) and one inhibitory (I), was used,

\[ V_a = \frac{V_{\text{max}}(S) + V_{\text{max}}(I)}{1 + (IC_{50}(A)^3) + V_{\text{max}}(I) + (IC_{50}(A)^3)} \]  

(2)

IC₅₀ here denotes the EC₅₀ of the inhibitory component. In some calculations basal was set as a constant to avoid a singular matrix.

**Chemicals** —The following agents were used: norepinephrine ((-)-arterenol bitartrate), isoprenaline ((-)-isoproterenol (+)-bitartrate), ionomycin (calcium salt), forskolin, Fura-2/AM, BAPTA, phorbol 12-myristate 13-acetate (PMA) (12-O-tetradecanoylphorbol-13-acetate), prazosin, yohimbine (hydrochloride), and dimethyl sulfoxide (for cell culture) all obtained from Sigma; CGP-12177 (CGP-12177A, from Ciba-Geigy), 3-isobutyl-1-methylxanthine (IBMX), 8-methoxysubstituted-3-isobutyl-1-methylxanthine (8-MM-IBMX), BAPTA-AM (all from Calbiochem), Ro 20-1724, cirazoline (hydrochloride) (from RBI, Natick), half-BAPTA from Molecular Probes, and OPC-3911 was a gift from Eva Degerman (Lund University). Stock solutions (10 mM) of agents used were normally made in 0.05% ascorbic acid (norepinephrine and isoprenaline) or in Dulbecco's modified Eagle's medium and stored at -80 °C. Ionomycin, forskolin, Fura-2/AM, prazosin, PMA, IBMX, 8-MM-IBMX, Ro 20-1724, and OPC-3911 were dissolved (1 to 100 mM) and kept in dimethyl sulfoxide. Up to 3% of dimethyl sulfoxide did not affect the cAMP accumulation. Routinely, the final concentration of dimethyl sulfoxide was kept <1%.

**RESULTS**

In the present experiments, precursors of brown adipocytes were isolated from mice and cultured under conditions leading to confluence on days 6 or 7. The cells are then fully differentiated, in that they contain numerous fat droplets and are able to express high levels of the brown fat-specific mitochondrial uncoupling protein (UCP1) in response to norepinephrine stimulation (3, 4). It is in cells like this that the unusual and distinctive kinetics for norepinephrine-induced UCP1 expression (3, 4, 6) and β₃-adrenergic receptor expression (11) have been observed, as well as the underlying similar kinetics of cAMP accumulation (6). The aim of the present investigation has been to obtain an explanation for these kinetics.

**An Unusual Norepinephrine Dose-Response Curve** —The characteristic dose-response curve for cAMP generation induced by norepinephrine (NE) is presented in Fig. 1A. As seen, in agreement with Ref. 6, the response did not adhere to simple Michaelis-Menten kinetics; rather, a very distinctive semi-bell-shaped dose-response curve was obtained. In control experiments, it was confirmed that the same relative relationships for the cAMP levels induced by 0.1, 1, and 10 μM NE were observed at any time point up to 90 min after NE stimulation (when the cAMP levels were still markedly elevated over unstimulated levels) and that the response peaked at ~20 min for all NE concentrations (not shown). Therefore, in the following experiments, the cAMP levels were determined 20 min after NE stimulation.

A possible explanation for a bell-shaped dose-response curve would be the presence of an inhibitory component, activated by high concentrations of NE. Following the theoretical suggestions of Rovati and Nicosia (5) and Equation 2 derived therefrom (see "Experimental Procedures"), we have analyzed in Fig. 1B the kinetics of the NE dose-response curve and found that it could be resolved into two hypothetical components: a stimulatory component with an EC₅₀ of 35 nM and an inherent ability to increase the cAMP level to 250% of that maximally observed experimentally, and an inhibitory component with a somewhat higher IC₅₀ value of 100 nM and an inherent ability to reduce the cAMP level by 200%. Through the interaction of these hypothetical components (the Resultant curve in Fig. 1B), a very good description of the experimental results (Fig. 1A) was obtained. Although it was thus possible with this theoretical analysis to describe the observation by introducing two counter-acting components, the question to be addressed was whether the two components postulated here had a true existence.

**The Nature of the Inhibitory Component** —In mature brown adipocytes, the stimulatory component of the cAMP response to NE is mediated via β₃-adrenergic receptors (6), and undoubtedly β₃-activation would be the stimulatory component in the above theoretical analysis.

The hypothetical inhibitory component could be mediated via the same β₃-receptors that mediate the stimulatory component or via other adrenergic receptors. The activated β₃-receptors in themselves could induce the inhibition, since released βγ-subunits of the Gₛ (or Gᵥ) protein can inhibit certain adenylyl cyclase subtypes (12–14). If this were to occur at high intensity of β₃-adrenoreceptor activation, it could explain the curve shape but it would not be possible to block only the inhibitory component by the use of a subtype-selective adrenergic antagonist. However, if other adrenergic receptors were involved, the correct subtype-selective adrenergic antagonist should be able to transform the biphasic dose-response curve into a simple, monophasic curve with the appearance of the hypothetical stimulatory component in Fig. 1B.

The obvious candidate for such an inhibitory receptor is the αₙ-adrenergic receptor that mediates its inhibitory effect directly on adenylyl cyclase via Gᵥ proteins. We therefore examined whether the αₙ-antagonist yohimbine would be able to eliminate the inhibitory component of the NE response. As seen in Fig. 1C, +Yoh, this was not the case: although there was a clear augmenting effect of yohimbine on the maximum response to NE, the inhibitory component was still very evident.

We therefore also examined whether the αₙ-antagonist prazosin could influence the dose-response curve. As seen in Fig. 1C, +Praz, this was indeed the case: prazosin not only doubled the maximal response (as did yohimbine) but it also eliminated the inhibitory component and converted the bell-shaped curve into a typical Michaelis-Menten curve.

In Fig. 1D, we have used the data from Fig. 1C to characterize the components predicted by the theoretical analysis in Fig. 1B. As seen, the experimentally obtained values, indicating a stimulatory β₃-component with an EC₅₀ of 34 nM and a large
To brown adipocyte cultures, 10 μM of the indicated agonist(s) were added 5 min before the indicated β₁-agonists and, where indicated, 1 μM cirazoline 1 min before the β₁-agonists. The cultures were harvested 20 min after β₁-agonist addition and cAMP levels were determined. Results are mean ± S.E. of five experiments (three experiments with yohimbine). In each series, the effect of 0.1 μM BRL-37344 or 1 μM CGP-12177 alone was set to 100% (which corresponded to 96 ± 11 pmol of cAMP per well for BRL-37344 and 68 ± 17 for CGP-12177). The data were analyzed for significant effect of further agents on the response to the β₁-agonist alone.

| Agonist(s) | BRL-37344 | CGP-12177 |
|-----------|-----------|-----------|
| No addition | <3        | <3        |
| β₁-Agonist | 100       | 100       |
| β₁-Agonist + prazosin | 97 ± 15% | 92 ± 8%   |
| β₁-Agonist + yohimbine | 78 ± 12% | 73 ± 6%   |
| β₁-Agonist + cirazoline | 44 ± 5%  | 32 ± 2%   |
| β₁-Agonist + prazosin + cirazoline | 103 ± 18% | 89 ± 10% |
| β₁-Agonist + yohimbine + cirazoline | 49 ± 13%  | 22 ± 2%  |

NS, not significant; Student’s paired t test.

*p < 0.1.

*p < 0.001.

*p < 0.05.

*p < 0.01.

increase in cAMP up to 231% of that normally seen, plus an inhibitory α₁-component with an IC₅₀ of 74 nm and inducing an inhibition of 170%, were very close to those predicted by the theoretical analysis. It was therefore concluded that the inhibitory component was not inherent to the β₁-receptors and was not mediated in the classical way via α₂-receptors. Instead, this inhibition was unexpectedly mediated via α₁-receptors.

To substantiate this interpretation, the dual nature of NE action was mimicked by combining a β₂-selective agonist (BRL-37344 or CGP-12177) with an α₁-selective agonist (cirazoline). In the brown adipocytes studied here, β₂-agonists induce a monophasic cAMP response (6) and they were used here at saturating concentrations. As expected, the β₂-agonists increased the cAMP level (Table I), and also as expected, this response was not affected by the α₁-antagonist prazosin. The α₂-agonist yohimbine in itself exhibited a tendency to an unexpected inhibitory effect. However, in accordance with the model presented above, activation of α₂-receptors with cirazoline led to a significant suppression of the cAMP accumulation induced by either of the β₂-selective agonists, which could be prevented by the α₁-selective antagonist prazosin. Thus, this result is in accordance with the implication that it is through α₁-receptors that the inhibitory component of the responses to NE is mediated.

The Response to Isoprenaline also Includes an Inhibitory α₁-Component—The agonist isoprenaline is generally considered to be a selective β₁-agonist. Thus, isoprenaline is not expected to stimulate α₁-receptors, and its dose-response curve would therefore not be expected to exhibit the α₁-inhibitory component identified above. However (Fig. 2A), although the maximal response to isoprenaline stimulation (66 pmol/well)
Dual Component Analysis of cAMP Accumulation in Brown Adipocytes

A cAMP accumulation

![Graph showing cAMP accumulation in response to isoprenaline](image)

B Component analysis

![Graph showing component analysis](image)

C Effects of α-antagonists

![Graph showing effects of α-antagonists](image)

D Experimental analysis

![Graph showing experimental analysis](image)

**Fig. 2.** Analysis of the dose-response curve for isoprenaline-induced cAMP accumulation in cultured mature brown adipocytes. 

A, dose-response curve for isoprenaline-induced cAMP accumulation. Cultured brown adipocytes were stimulated on culture day 6 with the indicated isoprenaline concentrations and cAMP levels were determined 20 min later. Points are mean ± S.E. of seven independent experiments. B, component analysis of the data in A. The data in A were analyzed as described in the legend to Fig. 1B, yielding a stimulatory component ($V_{\text{max}} = 110\%$, $EC_{50} = 8 \text{ nM}$, $h = 1.7$) and an inhibitory component ($V_{\text{max}} = 50\%$, $IC_{50} = 500 \text{ nM}$, $h = 1$). C, effect of α-antagonists on the dose-response curve for isoprenaline-stimulated cAMP accumulation. 10 μM yohimbine (Yoh) and/or prazosin (Praz) were added 5–7 min before the indicated concentration of isoprenaline and cAMP levels determined 20 min later. Each series, the effect of 0.1 μM isoprenaline alone was set to 100%, corresponding to 66 ± 9 pmol of cAMP/well. Points are mean ± S.E. of five experiments. D, component analysis of the experimental data in C. The ▽ points are identical to those for isoprenaline + prazosin in Fig. 1C but are here analyzed for adherence to simple Michaelis-Menten kinetics, yielding the indicated values. The Δ points were calculated as the difference between the isoprenaline points and the "isoprenaline + prazosin" points in Fig. 1C; they thus represent the inhibitory component. The inhibitory component was also analyzed for adherence to Michaelis-Menten kinetics, yielding the indicated values.

The nature of this PMA-elicited enhancement of cAMP accumulation, just as did isoprenaline (Fig. 2), is that these agonists to different degrees interact with α₁-receptors and that this can explain both the differences in shape of their dose-response curves and most of their apparently different efficacy. Based on the results presented in Figs. 1B and 2B, and Table I it was calculated that the inhibitory α₁-component led to a 74% reduction of the response to 1 μM NE and to a 39% reduction of the response to 1 μM isoprenaline; no inhibitory α₁-component was found in the response to CGP-12177 or BRL-37344.

Activation of Protein Kinase C Does Not Mediate the Inhibition—To understand the cellular mechanism of the α₁-inhibition, we proceeded to identify the second messenger responsible. As in other systems, stimulation of α₁-adrenoreceptors in brown fat cells produces two second messengers, inositol 1,4,5-trisphosphate (15, 16) and diacylglycerol; the inositol 1,4,5-trisphosphate releases Ca²⁺ from intracellular stores and [Ca²⁺], is increased (10, 17, 18), and the diacylglycerol activates protein kinase C (PKC) (19).

To investigate the possible participation of the diacylglycerol-PKC pathway, we stimulated the cultured adipocytes by the phorbol ester PMA (12-O-tetradecanoylphorbol-13-acetate); this had in itself no detectable effect (Table II). If PKC activation was responsible for the inhibitory effect of α₁-stimulation, PMA would be expected to inhibit selective α₁-agonist-induced cAMP accumulation, just as did α₁-stimulation (Table I). However, PMA did not inhibit, but rather enhanced the cAMP accumulation. The nature of this PMA-elicited enhancement of β₂-adrenoreceptor-induced cAMP accumulation was not further investigated but could conceivably be related to reported stimulation of certain adenylyl cyclase subtypes by PKC (20, 21). As a similar enhancing effect of PMA was observed also when the cells were stimulated with isoprenaline or NE (Table II), it is unlikely that this mechanism is actually activated when the cells are responding to adrenergic stimulation.

Conversely, we attempted to inhibit the PKC pathway during adrenergic stimulation. The addition of the PKC inhibitor...
chelerythrine (1 μM) before addition of NE or isoprenaline led to a
decrease in the responses to these agents by ~10%, i.e. the
opposite of what would be expected if PKC mediated the inhibitory
effect (not shown). Furthermore, long-term pretreatment with PMA, a treatment that is expected to desensitize PKC (22)
earlier indirectly shown to do so in these cells (10), did not
lead to an augmentation in the cAMP response to β-agonists
(Table II). Thus, the PKC pathway was not involved in media-
tion of the inhibitory α₁-component.

Ca²⁺ Is Necessary for the Inhibitory α₁-Response—To examine
whether the α₁-induced inhibitory effect was mediated via
an increase in cytosolic Ca²⁺ levels, we manipulated the intracellular Ca²⁺ response during adrenergic stimulation.

NE stimulation of cultured brown adipocytes led to an ele-
vated intracellular Ca²⁺ level (Fig. 3) (10). To investigate whether this was a necessary component in the inhibitory effect, we examined the effect of incubation conditions intended
to diminish the increase in [Ca²⁺]. Changing to a Ca²⁺-free
medium led to an approximate halving of the norepinephrine-
induced [Ca²⁺] response (Fig. 3); this was associated with an
increase in the level of norepinephrine-induced cAMP accumu-
lation (Table III). To further diminish the [Ca²⁺], levels, we
added the Ca²⁺ chelator BAPTA to the nominally Ca²⁺-free
medium. A further reduction in [Ca²⁺] response was observed
(Fig. 3), and an higher cAMP accumulation (Table III). To fully
eliminate the increase in cytosolic Ca²⁺ levels, we preincubated
the cells with the permeable Ca²⁺ chelator BAPTA/AM. As
seen, no NE-induced increase in [Ca²⁺], was then observed
(Fig. 3), and a further increase in NE-induced cAMP accumu-
lation was found. (The cell-permeable non-chelator “half-
BAPTA/AM,” which is not able to chelate Ca²⁺, had only a
limited effect on NE-induced [Ca²⁺], and did not influence the
NE-induced cAMP response (Fig. 3; Table III).) Thus, an in-
crease in [Ca²⁺], was clearly a necessary step in the mediation
of the inhibitory α₁-component.

Correspondence between Adrenergic Effects on [Ca²⁺], and on
Inhibition of cAMP Accumulation—If an increase in [Ca²⁺],
was fully responsible for the mediation of the inhibition, there
should be good correspondence between the relative ability of
adrenergic agonists to increase [Ca²⁺], and their ability to inhibit cAMP accumulation. In Fig. 4A, we exemplify the effect
of different adrenergic agonists on [Ca²⁺], levels in these cells.
In Fig. 4, B-D, dose-response curves for these effects are pre-
sented as a means from a series of experiments.

As seen, NE increased [Ca²⁺], levels markedly, with an EC₅₀
value of about 0.2 μM, and a maximal induction of more than
micromolar Ca²⁺ concentrations (Fig. 4B). At concentrations
>1 μM, isoprenaline had a clear, although modest, effect on
[Ca²⁺], levels (Fig. 4C) (this effect could be blocked by 1 μM
prazosin but not by propranolol, indicating that it was indeed
mediated via α₁-receptors; not shown). CGP-12177, however,
even at 10 μM, was fully without effect on [Ca²⁺], (Fig. 4A).

is clear that this relationship is fully parallel to the inherent
ability of each of these compounds to self-inhibit cAMP accumu-
lation through α₁-pathways: CGP-12177 had no such effect
(Table I), isoprenaline some (Fig. 2), and NE much greater (Fig. 1).

Effect of Increase in [Ca²⁺], on cAMP Accumulation—A fur-
ther, critical step in the demonstration that [Ca²⁺], mediates
the inhibitory component in the control of cAMP level in cul-
tured brown adipocytes was to experimentally increase the intracellular levels of Ca²⁺. Two methods were used for this. One was through activation of the receptor-mediated
pathway, i.e. through stimulation of α₁-receptors by the α₁-
selective agonist cirazoline. The other one was through the use
of the Ca²⁺ ionophore ionomycin.

Cirazoline, just as NE, led to a rapid increase in [Ca²⁺],
levels. The increase induced by both NE and cirazoline dis-
played Michaelis-Menten kinetics (Fig. 4, B and D), but the
EC₅₀ for cirazoline was, as expected, lower than that for NE (35
and 200 nM, respectively). The maximal level reached after NE
stimulation was consistently higher than that reached after
selective stimulation of the α₁-receptors with cirazoline; as
pure β-stimulation and an increase in cAMP with forskolin did
not elevate [Ca²⁺], (not shown), a complex interaction is indi-
In addition of 1 Fura/AM as described under “Experimental Procedures” before the adipocytes. A addition (mCGP-12177 or 0.1 coefficient h [Ca2+], except that different concentrations of NE were added, in different Fig. 4. Effects of agents on cytosolic Ca2+ levels in brown adipocytes. A, kinetics of increases in cytosolic [Ca2+] levels in response to different agents. Brown adipocytes were preincubated with Fura/AM as described under “Experimental Procedures” before the addition of 1 μM NE (0.1 μM where indicated), 1 μM cirazoline, 10 μM CGP-12177 or 0.1 μM ionomycin. For each experiment, the time of addition (heavy arrowhead) was set to 0. B, dose-response curve for the effect of NE on [Ca2+]. The experiments were principally performed as in A, except that different concentrations of NE were added, in different incubations. For each concentration, the maximal response to NE was measured, expressed as the increase over the basal value, i.e. as Δ[Ca2+]. The results are mean ± S.E. from five experiments. Data points were analyzed for best fit to a Michaelis-Menten equation (Equation 1), yielding an EC50 of 0.534 μM and a Vmax of 0.52 μM Ca2+ (h = 1.83). For estimation of the parameters of the ionomycin dose-response curve, the sum of a linear and a Michaelis-Menten function was used, i.e. Δ[Ca2+] = m [ionomycin] + Vmax/(1 + EC50/[ionomycin]). This yielded an EC50 of 0.11 μM ionomycin, a Vmax of 85 nM Ca2+, and an m value of 0.08. The pure linear approximation of the data is drawn on the figure as the curve indicated with an arrow. the S.E. is smaller than the size of the symbol.) C, dose-response curve for the effect of isoprenaline on [Ca2+]i. The experiments were principally performed as in B, except that different concentrations of isoprenaline were added. The results are mean ± S.E. from five experiments. Data points were analyzed for best fit to a Michaelis-Menten equation (Equation 1), yielding an EC50 of 0.38 μM isoprenaline and a Vmax of 0.89 μM Ca2+ (h = 1.14). D, dose-response curves for the effects of cirazoline and ionomycin on [Ca2+]i. The experiments were principally performed as in A and B. The Michaelis-Menten analysis yielded for cirazoline an IC50 of about 300 and 80 nM Ca2+, respectively. This difference can, however, be understood if the different kinetics of the Ca2+ responses to these agents are taken into account (Fig. 4A). The dose-response curves for the increases in Ca2+ (Fig. 4D) are based on the peak response. As the Δ[Ca2+]i for ionomycin is fairly stable with time, the peak level is a good approximation of the mean level during the 20-min incubation period. The S.E. is smaller than the size of the symbol.) D, dose-response curve for the effect of isoprenaline on [Ca2+]i. The experiments were principally performed as in B, except that different concentrations of isoprenaline were added. The results are mean ± S.E. from five experiments. Data points were analyzed for best fit to a Michaelis-Menten equation (Equation 1), yielding an EC50 of 0.38 μM isoprenaline and a Vmax of 0.89 μM Ca2+ (h = 1.14). D, dose-response curves for the effects of cirazoline and ionomycin on [Ca2+]i. The experiments were principally performed as in A and B. The Michaelis-Menten analysis yielded for cirazoline an IC50 of 0.38 μM isoprenaline and a Vmax of 0.89 μM Ca2+ (h = 1.14). D, dose-response curves for the effects of cirazoline and ionomycin on [Ca2+]i. The experiments were principally performed as in A and B. The Michaelis-Menten analysis yielded for cirazoline an IC50 of 0.38 μM isoprenaline and a Vmax of 0.89 μM Ca2+ (h = 1.14). D, dose-response curves for the effects of cirazoline and ionomycin on [Ca2+]i. The experiments were principally performed as in A and B. 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used for determination of cAMP levels. However, for cirazoline, the maximal level is clearly an overestimate of the mean elevation of [Ca^{2+}]_{i}. The mean level of D[Ca^{2+}]_i for the 20-min incubation may be estimated through integration of the area under the curves in Fig. 4 and is about a factor 3 lower than the maximal values (this proved to be valid for both cirazoline and NE). Thus, this kinetic factor of 3 may fully explain the apparent 3-fold difference between the cirazoline and ionomycin curves in Fig. 5, and an increase in [Ca^{2+}]_{i} is equally effective in inhibiting cAMP accumulation whether it originates via a receptor-mediated pathway or is induced by a Ca^{2+} ionophore.

Is the Increase in [Ca^{2+}]_{i} Induced by Norepinephrine Responsible for the Inhibition of cAMP Accumulation?—The relevant physiological question is really whether the observed increase in [Ca^{2+}]_{i} during adrenergic stimulation can quantitatively explain the inhibition observed in response to norepinephrine addition. We therefore tested whether the relationship between the increase in [Ca^{2+}]_{i} and the level of cAMP can be considered the same as that above, also when [Ca^{2+}]_{i} is increased through the innate stimulation elicited by NE. As seen in Fig. 6A, cirazoline, which may further increase [Ca^{2+}]_{i}, further inhibi-
addition of forskolin led to the expected increase in cAMP. When \([\text{Ca}^{2+}]\), was increased by the addition of 1 \(\mu\text{m}\) ionomycin, only a minor difference (\(-20\%\)) in the kinetic constants of cAMP accumulation was registered (Fig. 7A). This effect of \(\text{Ca}^{2+}\) on adenyl cyclase activity was thus too small to account for the large inhibition of cAMP accumulation observed above (\(-80\%\) according to Fig. 5B).

We therefore investigated whether the effect could be mediated via activation of a phosphodiesterase. Experiments were therefore performed in the absence of IBMX, i.e. when the phosphodiesterases were active. A different pattern was then observed (Fig. 7B). The maximal cAMP level achieved by forskolin was now only half that in the presence of phosphodiesterase inhibitor, implying the presence of a high basal phosphodiesterase activity in these cells (principally in accordance with Ref. 24)). When \([\text{Ca}^{2+}]\), was increased by the addition of 1 \(\mu\text{m}\) ionomycin, the cAMP level was further decreased by a factor of 2 (Fig. 7B). As the inhibitory effect of \(\text{Ca}^{2+}\) was only evident when the phosphodiesterases were active (compare Fig. 7, A and B), it was clear that activation of a phosphodiesterase was responsible for the inhibition of the cAMP accumulation by \(\text{Ca}^{2+}\) (and thus, by NE).

Which Phosphodiesterase Mediates the Effect?—Not all phosphodiesterases are \(\text{Ca}^{2+}\) sensitive. Out of at least seven distinct PDE gene families which generate more than 20 different isozymes, only the subtypes II, III, and IV have so far been positively identified in brown fat (25–27) but none of these PDEs are generally accepted to be \(\text{Ca}^{2+}\) stimulated; this is only the case for PDE I (CaM-PDE), which has not as yet been identified in brown adipose tissue. However, if CaM-PDE is the PDE responsible for the bell shape of the dose-response curve for NE, specific inhibition of this PDE should transform the bell-shaped curve to a normal Michaelis-Menten-type curve. The effect of several PDE inhibitors on the cAMP dose-response curve for NE was therefore studied.

Three selective PDE inhibitors, 8-MM-IBMX, OPC-3911, and Ro 20-1724, i.e. the inhibitors of subtypes I, III, and IV, respectively, were tested. At the concentrations used, the inhibitors inhibit only the indicated PDE subtypes (28–30). The inhibitors of PDE III and IV, i.e. OPC-3911 and Ro 20-1724, failed to convert the bell shape of the dose-response curve into normal kinetics, despite the fact that they considerably increased the maximal cAMP-response (Fig. 8A). Only 8-MM-IBMX, the selective inhibitor of the CaM-PDE (PDE-I), converted the dose-response curve to a normal Michaelis-Menten-type curve (Fig. 8A). This thus indicated that the inhibition was mediated via the PDE-I subtype.

Involvement of Calmodulin in the Mediation of the Inhibition—Mediation of the \(\text{Ca}^{2+}\) effect on PDE I occurs through calmodulin (31). Thus, if it is directly through the formation of a \(\text{Ca}^{2+}\)-calmodulin complex and its interaction with PDE-I that this phosphodiesterase is activated, antagonism of calmodulin should remove the inhibitory component of norepinephrine stimulation. In agreement with this, the potent antagonist of calmodulin, calmidazolium (IC\(_{50}\) \(~0.2 \mu\text{m}\) for CaM-PDE-activity (32)) at a concentration of 3 \(\mu\text{m}\) converted the bell-shaped curve to a Michaelis-Menten-type curve (Fig. 8B).

DISCUSSION

When stimulated with norepinephrine, brown adipocytes may exhibit non-Michaelis-Menten response kinetics in their responses. This is, e.g. evident for norepinephrine-induced UCP1 (3, 4) and \(\beta_1\)-adrenoreceptor (11) gene expression in cultured brown adipocytes from mice. These kinetics of gene expression are reflections of the norepinephrine-induced cAMP levels, which thus also display these characteristic kinetics (6). Thus, to understand the regulation of UCP1 and \(\beta_1\)-adrenore-
DUAL COMPONENT ANALYSIS OF cAMP ACCUMULATION IN BROWN ADIPOCYTES

The goal of the present investigation was to elucidate the mechanism behind the distinctive non-Michaelis-Menten kinetics of cAMP accumulation should be understood.

The effect of 0.1 μM NE alone (n = 3) in each experiment was set to 100%, corresponding to 25 ± 7 pmol of cAMP/well, and in different experiments, the following inhibitors were added 5–10 min before the indicated concentrations of NE: 100 μM 8-MM-IBMX (●); 0.5 μM OPC-3911 (▲); 15 μM Ro 20-1724 (▼). B. effect of the calmodulin antagonist calmidazolium on the dose-response curve for NE-induced cAMP accumulation. The effect of 0.1 μM NE alone in each experiment was set to 100% corresponding to 18 ± 2 pmol of cAMP/well. Results are mean ± S.E. from two experiments.

Thus, the uniqueness of the mechanisms reported here is related to the quantitative relationship between the two components: the inhibitory α1-pathway has a high capacity but also a relatively high EC50, compared with the stimulatory pathway, and through this, it shapes the characteristic dose-response curve and substantially influences the maximal cAMP levels reached.

The Large Effect of Δ[Ca2+]i—The inhibitory component was mediated via an increase in [Ca2+]i. The very low EC50 for Δ[Ca2+]i, for this effect, less than 100 nM, is remarkable. It is much lower than maximum norepinephrine-induced increases in [Ca2+]i, which may easily reach peak levels at least 10-fold higher, and there thus seems to be a high redundancy in the norepinephrine signal. However, considering the kinetics of the

cAMP accumulation. The effect of 0.1 μM NE with a relatively high EC50, explaining the bell-shaped kinetics as served. The inhibitory component was inherently much larger than normally observed for this effect, less than 100 nM, is remarkable. It is much lower than maximum norepinephrine-induced increases in [Ca2+]i, which may easily reach peak levels at least 10-fold higher, and there thus seems to be a high redundancy in the norepinephrine signal. However, considering the kinetics of the

The inhibitory component was mediated by Ca2+ stimulation of a cAMP phosphodiesterase. The interaction is therefore principally an example of negative cross-talk between intracellular signaling pathways, a phenomenon amply described over the last decades (36, 37). However, in its classical forms, cross-talk occurs as interaction between the signaling pathways of two different extracellular stimuli (hormones). Norepinephrine may mediate the stimulatory or the inhibitory component in different systems. Thus, cAMP levels induced via β-adrenergic stimulation may be reduced due to the effect of other hormones, which may act via activation of phosphodiesterase, as is the case for insulin (38). Conversely, when other hormones stimulate cAMP accumulation, norepinephrine may interact negatively, via α1-receptors and activation of a Ca2+/calmodulin-sensitive phosphodiesterase (39).

However, the present observations fall outside these types of classical cross-talk, because NE activates both the stimulatory adenyl cyclase component and the inhibitory component, and thus the cross-talk occurs between two pathways activated via the same activator. There is apparently only one earlier case in which norepinephrine has been implied to activate both pathways: heart cells (40). However, in those cells, the inhibitory α1 effect was small and the resultant curve was still a monophasic Michaelis-Menten relationship.
norepinephrine-induced \([Ca^{2+}]_i\) response, with its high negative time dependence, the high sensitivity may be understood as allowing for a sustained response: the phosphodiesterase system will remain activated until \(Ca^{2+}\) levels approaching basal have been reached. This high sensitivity of the response also means that agents with effects on \([Ca^{2+}]_i\), that seem trivially small may still be of significance for the outcome. The effective increase, a \(\Delta[Ca^{2+}]_i\), of <100 nm, seems low also when related to the basal level of \([Ca^{2+}]_i\), which has been estimated in this and most earlier investigations to be in the order of 200 nm. A technical complication may perhaps explain this. In the present investigation, as in many others, the \(R_{\text{fin}}\) used for the calibration of the fluorescence signal necessary for determination of \([Ca^{2+}]_i\), has been established with Mn\(^{2+}\), which functions to eradicate the fluorescence. This may lead to some overestimation of the basal level, and the relative increase in \([Ca^{2+}]_i\), may therefore be higher than anticipated from the values given here.

The high sensitivity of the system to \(\Delta[Ca^{2+}]_i\), may also explain the somewhat unexpected results concerning isoprenaline. Isoprenaline is routinely used in receptor and receptor response studies as a selective \(\beta\)-agonist, and it is generally anticipated that the response does not include effects of \(\alpha\)-receptors. The data presented here (Figs. 2 and 4) clearly demonstrate, however, that at commonly used isoprenaline concentrations, \(\alpha\)-pathways are stimulated in brown adipocytes. In this respect, isoprenaline-induced responses are therefore principally different from those of selective \(\beta\)-agonists, for which we see no indication of an \(\alpha\) effect. It is possible that this \(\alpha_1\)-component may explain several recent observations on brown fat cells where the response to isoprenaline deviated from that to selective \(\beta_2\)-stimulation or direct adenyl cyclase activation (41–43). That isoprenaline seems to influence the \(\alpha_1\)-pathway to a larger extent in brown fat cells than in many other tissues may be due to the very high level of expression of \(\alpha_1\)-receptors in brown adipose tissue, compared with the levels in other tissues (44). If this is paralleled by a similar high level of \(\alpha_1\)-receptors, the functional affinity of brown fat cells for \(\alpha_1\)-stimulation would be high, and agents with only a small \(\alpha_1\) activity, such as isoprenaline, would manifest their \(\alpha_1\) action particularly in brown adipocytes.

**Physiological Relevance of the Inhibition**—An important difference between brown adipocytes in situ and those studied here in culture is that the cultured cells are naive with respect to adrenergic stimulation, whereas the brown adipocytes in situ are exposed to chronic adrenergic stimulation of alternating intensity. However, this may even accentuate the situation described here, because chronic adrenergic stimulation increases the density of the \(\alpha_1\)-receptors that mediate the inhibitory component (both gene expression levels (44, 45) and receptor number (46, 48)), whereas expression of the stimulatory \(\beta_1\)-receptors is down-regulated (47, 48), as is the activity of some of the mediating steps (49).

Functionally, all processes that are rate-limited by cAMP levels will be expected to show bell-shaped dose-response curves, but this does not mean that all responses mediated via an increase in cAMP levels will show this pattern. Thus, processes saturated at fairly low cAMP levels will display monophasic kinetics even if the underlying cAMP response is biphasic. This may, e.g., be the case for thermogenesis where saturation apparently occurs already at levels of cAMP only 50% of those maximally induced by norepinephrine (24).

The \(\beta_2\)-adrenergic receptor has as one of its features, in comparison to \(\beta_1/\beta_2\)-receptors, the absence in its primary structure of the serine residues that are involved in classical desensitization (50). Although this inability to classically desensitize was originally considered the feature of choice of \(\beta_2\)-receptors, it would seem that concerning this receptor, nature utilizes two alternative pathways to achieve a functional desensitization: both an intensive down-regulation of \(\beta_2\)-receptor gene expression (47, 48), and the activation of an \(\alpha_2\)-mediated inhibitory pathway, as demonstrated here.

\(\alpha_1/\beta_2\)-Interaction in Brown Adipose Tissue Function—The function of brown adipose tissue is highly regulated via \(\beta\)-adrenergic processes, mediated through an increase in cytosolic cAMP levels. This is so concerning both the acute thermogenic response and stimulation of cell proliferation, apoptosis, and cell differentiation. Thus, based solely on the data reported here, \(\alpha_1\)-stimulation should be associated with a suppressing effect on the function of brown adipose tissue.

However, a series of observations indicate a positive or synergistic interaction between \(\alpha_1\)- and \(\beta\)-stimulation. Thus, \(\alpha_1\)-stimulation enhances the ability of a given level of cAMP to induce thermogenesis in the cells (33), and \(\alpha_1\)-stimulation may, at least in brown fat cells from certain species, have some thermogenic effects in itself (51). Furthermore, and perhaps most importantly, \(\alpha_1\)-stimulation has additive or synergistic effects on the gene expression of a series of very significant genes for brown adipose tissue function, such as c-fos (10), thymidine 5'-deoxynucleoside (52), lipoprotein lipase (53), and the uncoupling protein (UCP1) itself (3, 4).

Thus, presently, it can only be concluded that cells of brown adipose tissue have an elaborate system for interaction between \(\beta\)- and \(\alpha_1\)-adrenergic signals, some positive, some apparently negative. We have clearly still not reached a sufficient depth of knowledge to allow us to understand the intricacies of the complex network found in this cell system, unique in its dependence upon adrenergic stimulation for cellular growth and function.

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