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CATECHOLAMINE-INDUCED LIPOLYSIS: ONLY VIA CYCLIC AMP?

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Abstract—Okuda *et al.* (*J. Biochem.* **75**, 131 (1974)) recently showed that lipolysis in lipid micelles (LM) prepared by hypotonic treatment of adipocytes was stimulated by adrenaline. The authors suggested that adrenaline acted by a direct promotion of the reaction between triglycerides and lipase, circumventing the adenylate cyclase system. In the present study we confirmed that adrenaline (as well as other hormones) stimulated lipolysis in LM. However, the release of free fatty acids (FFA) was accompanied by a measurable accumulation of cyclic AMP. Furthermore, the β-adrenolytic (−)-bupranolol caused a competitive inhibition of catecholamine-induced lipolysis, while the effects of ACTH remained unchanged. The LM preparation performed as described by Okuda *et al.* was found to be highly contaminated with intact fat cells. Additional hypotonic treatment of LM stepwise reduced the content of adipocytes, accompanied in a parallel pattern by a decrease of hormone-induced cyclic AMP accumulation and FFA release to the limit of detection. Our results show that the intact fat cells remaining in LM preparations were responsible for the effects seen after stimulation of LM with hormones.

INTRODUCTION

A hormone-sensitive lipase, first described by Rizack [1] represents the key enzyme in the mobilization of free fatty acids from adipose tissue. Experiments with intact adipose tissue [2], as well as with isolated adipocytes [3], and adipose tissue homogenates [4] revealed the physiological importance of the effects of individual hormones on the release of free fatty acids (FFA) and glycerol from endogenous substrate. It is generally accepted that hormone-induced lipolysis in adipose tissue is mediated through an activation of adenylate cyclase with subsequent formation of adenosine 3′,5′-monophosphate (cAMP) which is responsible for the activation of cAMP-dependent protein kinase. This, in turn, catalyzes the phosphorylation of inactive hormone-sensitive triglyceride lipase to give its active form [5, 6]. However, Okuda and coworkers [7, 8] have recently shown that lipolysis was stimulated by adrenaline, dibutyryl-cAMP and theophylline in lipid micelles isolated from adipocytes, but not by addition of cAMP. On account of the lack of added cAMP to stimulate lipolysis in lipid micelles [7, 8], the authors suggested that adrenaline acted by promoting the reaction between triglyceride lipase and its substrates and not by activating lipolysis via adenylate cyclase. A lipolytic effect of catecholamines circumventing adenylate cyclase appeared to be interesting enough to reinvestigate the mode of action of adrenaline and other hormones in lipolysis of so-called lipid micelles. A preliminary report of some of this work has been presented at the Spring Meeting of the Deutsche Pharmakologische Gesellschaft [9].

MATERIALS AND METHODS

Chemicals were obtained from the following sources: Bovine serum albumin (BSA) (fraction V, Serva Feinbiochemica) was purified by charcoal treatment according to Chen [10]; crude bacterial collagenase (Worthington Biochemical Corporation); adenosine [U-^{3}H]-3′,5′-cyclic phosphate, sp. act. 24.1 Ci/m-mole (NEN Chemicals GmbH); cyclic 3′,5′-AMP (Boehringer Mannheim); (−)-noradrenaline hydrochloride (Schuchardt GmbH); ACTH (Synacthen®, CIBA); bupranolol (Betadrenol®, Sanol–Arzneimittel Dr. Schwarz GmbH Monheim); adrenaline (Farbwerke Hoechst AG); all other chemicals were analytical grade or best commercially available.

Fat cell preparation. Isolated fat cells were prepared according to Rodbell [3] from epididymal adipose tissue of Wistar rats (220–280 g), fed *ad lib.* The cell preparation was diluted to 6–8 × 10^5 cells/ml with Krebs–Ringer phosphate buffer (pH 7.4) containing 2% of bovine albumin (referred to as Step 1).

Preparation of lipid micelles. After counting the fat cells in a 5 μl sample of a 80-fold dilution as hanging drops on a microscopic slide, the fat cells were suspended in a hypotonic lysing medium (5 mM Tris–HCl buffer, pH 7.4) at room temperature [11]. The suspension was mixed by slowly inverting the plastic centrifuge tube several times (twenty five times within 2 min) and centrifuged at 200 *g* for 2 min. The supernatant fraction below the fat layer was withdrawn through a plastic tube and replaced by an equal volume of 5 mM Tris–HCl buffer. The procedure of mixing by inversion and centrifugation was repeated four times, according to Okuda and Fujii [11], referred to as Step 2. Additional washings up to nine times are designated as Steps 3–6 in text or figures. After each step, an aliquot of the micelle suspension was diluted 40-fold and 5 μl samples were quickly examined (as hanging drops on a microscopic slide) for cell content.

Lipolysis in adipocytes and lipid micelles. After each step, aliquots (250 μl) of the suspensions were added to equal volumes of Krebs–Ringer phosphate buffer

(pH 7.4; 37°) containing 2% bovine albumin. In the presence or absence of lipolytic agents, the samples were incubated in plastic vials at 37°. The incubation was terminated by adding 0.5 ml of a copper reagent according to Noma *et al.* [12], containing 2.0 M Cu $(NO_3)_2 \cdot 3H_2O$. To 400-μl aliquots, 1.0 ml of chloroform–heptane–methanol (49:49:2 by vol) was added. The samples were vigorously shaken, kept at 0° for 1 hr, mixed again and were centrifuged at 12,000 *g* for 2 min. 0.7-ml Aliquots of the upper organic phase were transferred to test tubes and mixed with 250 μl DDC-reagent (0.1% sodium diethyl dithiocarbaminate in (2)-butanol) according to Duncombe [13]. Extinction was measured at 436 nm against the reagent blank. Standard curves with palmitic acid were run with each experiment. Lipase activity was expressed as μeq FFA/ml micelle preparation/2 hr.

Determination of cyclic AMP accumulation. Incubation of adipocytes and lipid micelle suspensions in order to determine cAMP levels was similar to the incubation for lipolysis and performed at each step. The usual incubation time was 5 min. The incubation (0.5 ml) was terminated by adding 0.5 ml 10% trichloroacetic acid (TCA). The precipitated material was removed by centrifugation (2 min at 12,000 *g*). Two-hundred μl aliquots of the supernatant were extracted three times with 1 ml water-saturated diethyl ether in order to remove TCA. After lyophilising, the samples were dissolved in 200 μl sodium acetate buffer (pH 4.0, 50 mM). Cyclic AMP levels were determined according to the method of Gilman [14]. It was ascertained that none of the tested compounds interfered with the assay of cAMP. Results were expressed as pmoles cAMP per ml micelle preparation per 5 min.

Staining and counting adipocytes and lipid micelles. Using the fluorescent dye acridine orange [15], adipocytes and micelle suspensions were stained in order to determine the contamination of lipid micelle preparations (LM) with intact adipocytes. The stained suspensions were observed in a Zeiss fluorescence microscope at 50-fold magnification. The differentiation between lipid micelles, normal living cells and metabolically inactive cells is facilitated by fluorescence at different wavelengths [16]. Lipid micelles show a pale green fluorescence, the nuclei of living cells show a bright green to yellow fluorescence, while the cell membranes are yellow. Nuclei of metabolically inactive (dying) adipocytes show a fluorescence ranging from orange to red.

RESULTS

In Fig. 1, the effect of several lipolytic agents on lipolysis in LM, prepared up to Step 2, are shown as dose response curves, obtained from the same LM preparation. We could confirm the observation [7] that adrenaline stimulated lipolysis in this LM preparation. Noradrenaline and ACTH showed to be even more powerful lipolytic agents than adrenaline. Maximal values for lipolysis (expressed as μeq FFA/ml LM × 2 hr), stimulated with ACTH were reached at a concentration of 50 nM ACTH. In comparable experiments with isolated fat cells (Step 1), the concentrations of the stimulants necessary to elicit half-maximal or maximal effects were almost identical [17,19].

A time-course showed that lipolysis in LM (Step 2) incubated with adrenaline (2 μM) proceeded continuously for at least 2 hr. The release of FFA, however, was accompanied by a measurable accumulation of cAMP. In contrast to cAMP time-courses performed in adipocytes [18], a time-course for LM did not show a maximal cAMP level within 5–10 min with a subsequent return to control levels after approximately 30 min of incubation. The cAMP accumulation in LM did not reach maximal values until 60 min of incubation time (15 pmoles cAMP/ml LM).

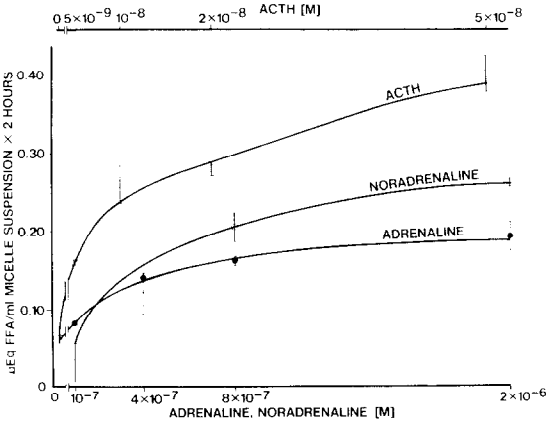

Fig. 1. Effects of various concentrations of ACTH, adrenaline and noradrenaline on the lipolysis of a single LM preparation, prepared up to Step 2. 1 ml micelle preparation is equivalent to 0.13 g fat tissue. The LM preparation contained about 98,000 intact cells/ml. Each point represents the mean ± S.E.M. of two experiments performed in duplicate. For details see Methods.

The accumulation of cAMP in LM (Step 2) after incubation in the presence of noradrenaline prompted us to examine LM by fluorescence microscopy, showing micelle preparations which were highly contaminated with intact adipocytes. Varying with different preparations, 20–40 per cent of the number of adipocytes from Step 1 was found. Additional hypotonic treatment (Steps 3–6) of LM, stepwise reduced contamination with intact cells to 7 per cent counted in Step 1. This reduction was accompanied by a significant decrease of cAMP accumulation and FFA release induced by 4 μM noradrenaline, which is supramaximal with respect to both parameters in adipocyte suspensions (Fig. 2). No stimulation of lipolysis could be obtained at the lowest contamination rate we finally achieved by nine washings corresponding to Step 6.

To obtain supporting evidence that the lipolytic effects of catecholamines in LM represent an interaction with adrenergic β-receptors located at the fat cell membrane, experiments were done to show an antagonism by β-adrenolytics. Bupranolol caused a dose-dependent inhibition of noradrenaline-stimulated lipolysis at concentrations ranging from 0.004 μM to 4 μM. Lipolysis stimulated by 4 μM noradrenaline was inhibited completely by bupranolol at an equimolar concentration. No interference by bupranolol was seen using ACTH as the stimulant (results not shown). A double reciprocal plot (Fig. 3) according to Lineweaver and Burk [20], which also allows an estimation of the 'apparent affinity' (K_m) of the stimulant, clearly showed a competitive pattern in the presence of bupranolol. The K_m of noradrenaline was

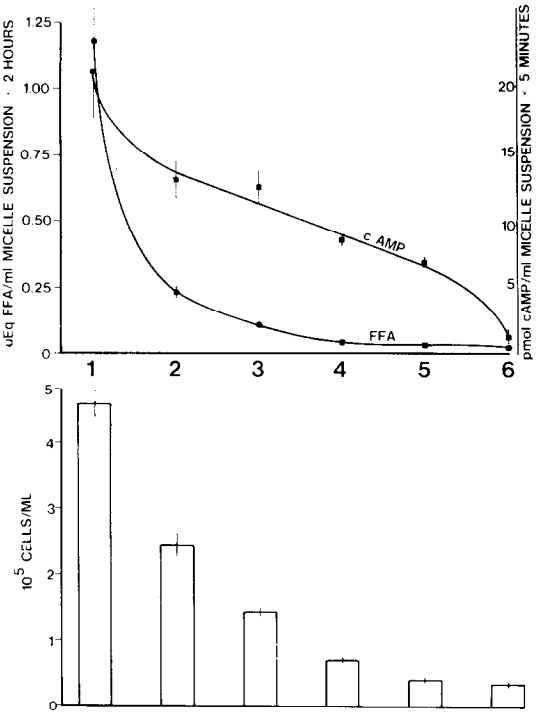

Fig. 2 Effects of stepwise hypotonic treatment on cAMP accumulation, lipolysis and fat cell content of LM preparations. Isolated fat cells (Step 1), were further treated by hypotonic washings as described in Methods. Numbers on the abscissa refer to the steps defined in Methods. Incubations were performed in the presence of 4 μM noradrenaline. Determinations are the means $\pm$ S.E.M. of 3 experiments performed in duplicate. For details see Methods.

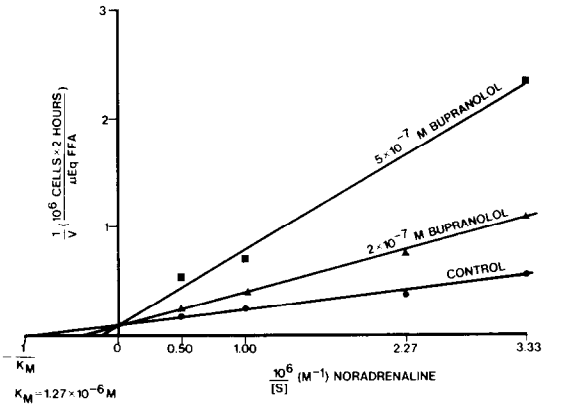

Fig. 3 Double reciprocal plot of the inhibition of lipolysis by the β-adrenolytic (−)-bupranolol. LM, prepared up to Step 2 were incubated in the presence of 0.3–2 μM noradrenaline. The lipolytic effect (ordinate) was calculated by the cell numbers of the LM used ($1.5 \pm 0.24 \times 10^5$ cells/ml). Each point represents the mean of 3 experiments performed in duplicate. For details see Methods.

found to be 1.27 μM which agrees with the value found in intact adipocytes [19]. The K_i estimated for bupranolol was 0.13 μM, which was also found in intact fat cells [19] or even in whole fat pads [26].

DISCUSSION

Our results confirm previous observations [7,8] that adrenaline induces lipolysis in so called lipid micelles (LM), prepared according to Okuda and Fujii [11]. In addition we found that noradrenaline as well as ACTH, both known to activate lipolysis via adenylate cyclase by binding to specific receptors on the outer cell surface (for review see [21]), stimulated lipolysis in LM even to a greater extent. In their discussion Saito *et al.* [22] briefly mentioned, without giving experimental details, the presence of 30 per cent adenylate cyclase activity in LM, as compared to adipocytes, indicating at least the presence of membrane-like structures. The authors propose an interesting 'first messenger' hypothesis for the lipolytic action of adrenaline in LM preparations, without mediation by activation of adenylate cyclase and subsequent activation of protein kinase. Their arguments are based on the following observations: (1) the extent of adrenaline-induced lipolytic activity in micelles and adipocytes was equal; (2) no increase of protein kinase activity was observed during stimulation of lipolysis in LM by adrenaline; (3) cAMP (4 mM) added to LM did not induce lipolysis in contrast to dibutyryl-cAMP (4 mM), although the former was found to penetrate adipocyte cell membranes as readily as the dibutyryl derivative [7].

Our data, however, show an accumulation of cAMP concomitant to the FFA release in LM after the addition of hormones. Lipolysis seen with noradrenaline was inhibited competitively by the β-adrenolytic bupranolol, as shown before in adipocyte suspensions [19]. If K_m of noradrenaline and K_i of the inhibitor were calculated by the number of cells found in the LM used, values almost identical with those found in adipocytes were obtained. Furthermore, it has been shown in adipocytes [19] or plasma membranes [24] that the formation of cAMP induced by catecholamines is also blocked competitively by β-adrenolytics. The concentrations of the β-adrenolytic used were sufficiently low to indicate an interaction with noradrenaline receptors at the cell membrane and to exclude 'unspecific' effects of β-adrenolytics which occur beyond the formation of cAMP and definitely show non-competitive antagonism [23].

In our hands, however, lipolysis found in LM was reduced to the limit of detection by additional hypotonic washings beyond the steps described by Okuda and Fujii [11]. Moreover, (fluorescence-) microscopy showed that the LM was contaminated with intact adipocytes.

Thus, it appears that under the described conditions the remaining intact cells in LM preparations, previously considered as contamination of LM, are at least in our studies responsible for all the effects seen after stimulation with hormones.

A definite answer to the question whether catecholamines are unable at all to stimulate lipolysis by circumventing the adenylate cyclase system requires more refined studies. Based on findings with ACTH and some of its derivatives, Schwyzer [25] discusses the possibility that an ACTH-induced increase of cAMP in adipocytes could be a rather parallel than a causative event with regard to lipolysis. To our knowledge, similar studies with catecholamines have yet to be performed.

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