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EFFECTS OF BENZYDAMINE ON THE LIPOLYTIC SYSTEM OF FAT CELLS

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Abstract—Benzydamine dose-dependently increased cyclic 3′,5′-AMP levels in isolated fat cells and competitively inhibited phosphodiesterase activity (K_i = 1·1 mM). ATP levels of isolated cells and cyclic 3′,5′-AMP-dependent protein kinase activity were not affected. However, benzydamine caused a dose-dependent inhibition of lipolysis in isolated fat cells stimulated by norepinephrine or dibutyryl cyclic 3′,5′-AMP. The enhancement of cyclic 3′,5′-AMP formation may be due to the local anaesthetic properties of benzydamine. The antilipolytic effect appears to result from the direct inhibition of hormone sensitive triglyceride lipase.

Recent studies from this laboratory showed that the antiphlogistic drug benzydamine is able to reduce mucopolysaccharide synthesis in fibroblast tissue cultures by inhibition of adenylate cyclase [1]. This effect was also visible, when cells were stimulated by prostaglandin E_1, even though the stimulation of cyclic 3,′5′-AMP formation and mucopolysaccharide secretion were not completely abolished by the drug.

Other non-steroidal antiphlogistic drugs like salicylates, phenylbutazone, and mefenamic acid affected lipolysis of isolated fat cells. The mode of action of these drugs on the adenylate cyclase system appeared to be identical in fat cells and fibroblasts, since the antilipolytic or lipolytic effect in fat cells as well as the effects on mucopolysaccharide secretion in fibroblasts closely correlated with actions on different components of the adenylate cyclase system [2, 3, 4]. The present study describes the influence of benzydamine on the cyclic 3′,5′-AMP dependent lipolytic system of epididymal fat cells. A preliminary account of some of this work has been presented [5].

MATERIALS AND METHODS

Materials were obtained from the following sources: Benzydamine-HCl (Kali-Chemie, Hannover); bovine serum albumin, fraction V, powder (Armour Pharmaceutical Co.); bacterial collagenase and lyophilized firefly lanterns (Worthington Biochemical Corp.); *l*-norepinephrine bitartrate (NE) (Fluka AG); adenosine triphosphate (ATP), adenosine 3′,5′-monophosphate (cyclic 3′,5′-AMP) and dibutyryl cyclic 3′,5′-AMP (Db-3′,5′-AMP) (Boehringer, Mannheim); ^{3}H-adenine (6 Ci/m-mole) and ^{3}H-cyclic 3′,5′-AMP (24·1 Ci/m-mole) (New England Nuclear); ^{32}P-cyclic 3′,5′-AMP (420 mCi/m-mole) and γ-^{32}P-ATP (20·3 Ci/m-mole) (Amersham Buchler, Braunschweig); histone (Schuchardt, München). All other chemicals were reagent grade from the usual commercial sources.

Male Wistar rats (170–220 g) with free access to food and water were used in all experiments with isolated fat cells.

Procedures for the determination of lipolysis, ATP levels, phosphodiesterase, and protein kinase activity have been described earlier [2, 4]. Cyclic 3′,5′-AMP levels in isolated fat cells were determined according to the method of Gilman [6]. Results were expressed as nmoles cyclic 3′,5′-AMP per 10^6 cells.

Lipase activity in adipose tissue homogenates was assayed by measuring free fatty acids (FFA) according to Duncombe [7] and Noma *et al.* [8] with some modifications. Wistar rats (250–300 g) were fasted 24 hr prior to sacrifice in order to minimize lipoprotein lipase activity [9]. Epididymal fat pads were preincubated for 2 hr in 5 ml of Krebs–Ringer phosphate buffer (pH 7·4), containing 3% bovine serum albumin, since this procedure eliminates possible interferences by the action of lipoprotein lipase [9]. Norepinephrine (NE), when present, was added 5 min before the end of the preincubation period. Fat pads were homogenized in a glass tissue grinder containing 10 vol of 40 mM phosphate buffer (pH 6·8) with 5 mM EDTA and 2% bovine serum albumin. The homogenate was subjected to ultrasonication for 5–10 sec (Branson Sonifier B 12, microtip, energy level 100 W) and 100 μl aliquots were quickly transferred into the incubation vials and incubated at 30°. Benzydamine concentrations tested were dissolved in buffer and placed in the incubation vials as drops of 25 μl just before addition of the homogenate. Since the reaction was linear only for 30–40 min, incubations were terminated after 30 min by adding 0·25 ml of copper reagent and 1 ml of extraction solvent [8]. The samples were mixed vigorously, kept in an ice bath for 1 hr, mixed again and centrifuged for 2 min at 12,000 *g*. 0·7 ml aliquots of the upper organic phase were transferred to other test tubes and mixed with 0·1 ml DDC reagent (0·1% sodium diethyldithiocarbaminate in (2)-butanol). Extinction was measured at 436 nm against a reagent blank. Standard curves with palmitic acid were run with each experiment. After substraction of zero-time values from the experimental values lipase activity was expressed as μEq FFA per g wet wt of adipose tissue per 30 min.

RESULTS

Benzydamine dose-dependently inhibited lipolysis of isolated fat cells, maximally stimulated either by

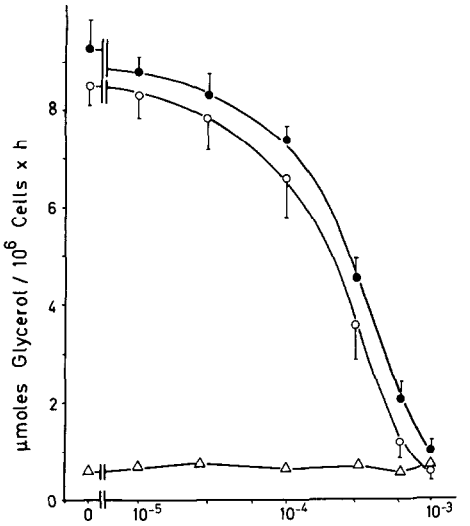

Fig. 1. Dose–response curve of benzydamine on lipolysis of isolated fat cells. Lipolysis was stimulated by either 1 µM NE (○——○) or 3 mM Db-3′,5′-AMP (●——●); basal lipolysis (△——△). Incubations were performed for 1 hr at 37°. Each value represents the mean ± S.E.M. of 4–7 experiments performed in duplicate.

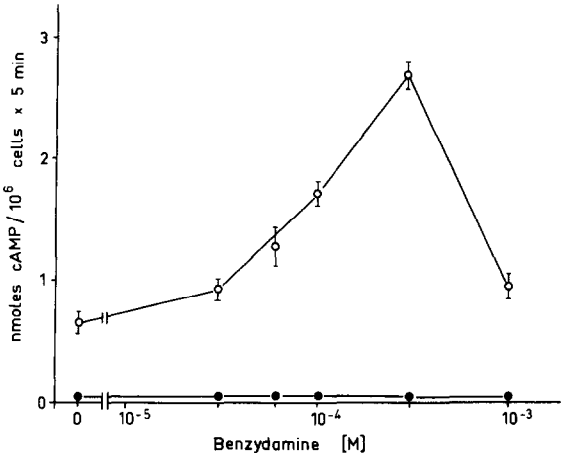

Fig. 2. Effect of benzdamine on cyclic 3′,5′-AMP levels in fat cells in the presence and absence of 1 µM NE. Results are expressed as nmoles cyclic 3′,5′-AMP per 10^6 cells during 5 min incubation. Each value represents the mean ± S.E.M. of 5 experiments performed in duplicate.

10^{-6} M NE or 3×10^{-3} M Db-3′,5′-AMP, whereas basal glycerol production was not affected (Fig. 1). Half-maximal inhibition occurred at 0·3–0·4 mM benzydamine.

In the same dose-range the drug did not affect basal levels of cyclic 3′,5′-AMP, but doubled or almost tripled cyclic 3′,5′-AMP levels in the presence of 1 µM NE after 5 min incubation at 0·1–0·4 mM (Fig. 2). Higher concentrations caused a rapid fall in cyclic 3′,5′-AMP levels.

These findings were confirmed in experiments on the time-course of cyclic 3′,5′-AMP levels after stimulation of the cells by 1 µM NE or 1 µM NE plus 0·5 mM theophylline in the presence and absence of 0·3 mM benzydamine (Fig. 3). The drug potentiated the effect of NE on cyclic 3′,5′-AMP levels without preventing the secondary decline after 10 min. Since diluted cell suspensions were used in these experiments (20,000–50,000 cells/ml), addition of theophylline to NE only doubled the cyclic 3′,5′-AMP levels compared with NE alone [10]. However, an identical potentiation of the stimulants was obtained by the drug under these conditions.

The effect of benzydamine on cyclic 3′,5′-AMP levels cannot be explained by higher levels of ATP within the cells, since benzydamine alone did not influence intracellular ATP levels in the dose range between 0·01 and 1 mM. Furthermore, the decline in ATP levels following stimulation of the cells was in good correlation with the observed formation of cyclic 3′,5′-AMP (Table 1), indicating that the drug did not interfere with the conversion of ATP into cyclic 3′,5′-AMP at the catalytic site of the adenylate cyclase.

Since an increase of cyclic 3′,5′-AMP levels may be caused by inhibition of phosphodiesterase, the in-

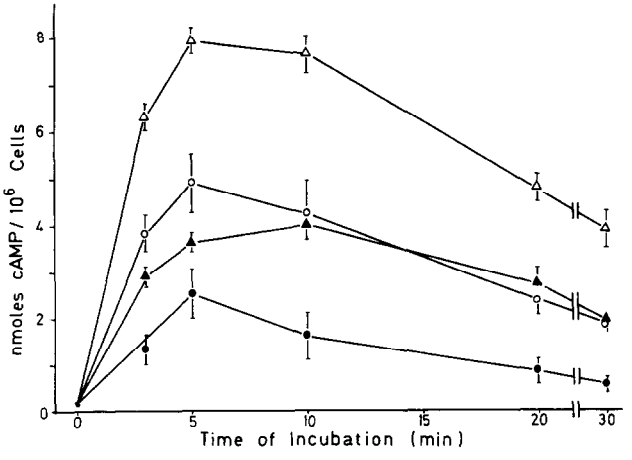

Fig. 3. Effect of 0·3 mM benzydamine (open symbols) on the time course of cyclic 3′,5′-AMP formation in isolated fat cells stimulated by 1 µM NE (circles) or 1 µM NE plus 0·5 mM theophylline (triangles). Incubations were performed at 37°. Each value represents the mean ± S.E.M. of 5 experiments performed in duplicate.

Table 1. Effect of benzydamine on ATP levels in fat cells following stimulation of the cells by 1 μM NE or 1 μM NE plus theophylline (TE)

	nmoles ATP/10^6 Cells, measured at various incubation periods		
	0 min	5 min	10 min
Control	17·29 ± 3·07	—	17·18 ± 2·93
0·3 mM Benzydamine	17·32 ± 3·10	—	17·26 ± 3·25
1 μM NE	16·77 ± 3·15	12·22 ± 2·84	10·44 ± 2·34
0·3 mM Benzydamine + 1 μM NE	17·43 ± 3·15	5·32 ± 1·65	4·79 ± 1·47
1 μM NE + 0·5 mM TE	17·76 ± 3·24	10·33 ± 2·38	7·47 ± 1·11
0·3 mM Benzydamine + 1 μM NE + 0·5 mM TE	17·18 ± 3·05	4·05 ± 1·23	3·66 ± 1·03

Incubations were identical with those described in Fig. 3. Each value represents the mean ± S.E.M. of 5 experiments performed in triplicate.

fluence of benzydamine on the activity of this enzyme was tested. The compound showed an inhibitory effect both in the low (Fig. 4) and in the high K_m-range of phosphodiesterase activity. The inhibition was competitive in accordance with an inhibitor constant of $K_i = 1{\cdot}1$ mM as determined from a Dixon-plot. Identical findings were obtained with the high K_m enzyme.

To elucidate the discrepancy between inhibition of lipolysis and enhancement of cyclic 3′,5′-AMP levels, the influence of benzydamine on cyclic 3′,5′-AMP-dependent and independent protein kinase and lipase activity was studied. The compound did not impair the binding between cyclic 3′,5′-AMP and cyclic 3′,5′-AMP-dependent protein kinase measured as described earlier [2]. Furthermore, the function of this enzyme in phosphorylating histone was not affected by 0·05–1 mM benzydamine (Table 2).

As shown in Fig. 5, benzydamine caused a dose-dependent inhibition of lipase activity, when FFA production of adipose tissue homogenates was measured.

Half-maximal inhibition occurred in the same concentration range as that found in lipolysis of isolated fat cells. Five min preincubation of fat pads with 1 μM NE prior to homogenization produced an increase in lipase activity of about 80%, indicating that hormone sensitive triglyceride lipase activity is measured by this test system.

DISCUSSION

The results described in this study show that benzydamine has two main actions on the lipolytic system of intact fat cells: (1) inhibition of lipolysis, (2) increase of cyclic 3′,5′-AMP levels. Benzydamine inhibits NE-stimulated as well as Db-3′,5′-AMP-stimulated lipolysis. Therefore, the essential metabolic lesion appears to be beyond the formation of cyclic 3′,5′-AMP by adenylate cyclase. No effect was found on the ATP content of the fat cells, on the binding of cyclic 3′,5′-AMP to the cyclic 3′,5′-AMP-dependent protein kinase and on the function of the catalytic subunit of the enzyme when phosphorylation of histone was determined. In our experiments protein kinase from bovine diaphragm muscle was used. It is justifiable to correlate these findings to protein kinase from adipose cells, since protein kinases from various tissues have almost identical kinetic para-

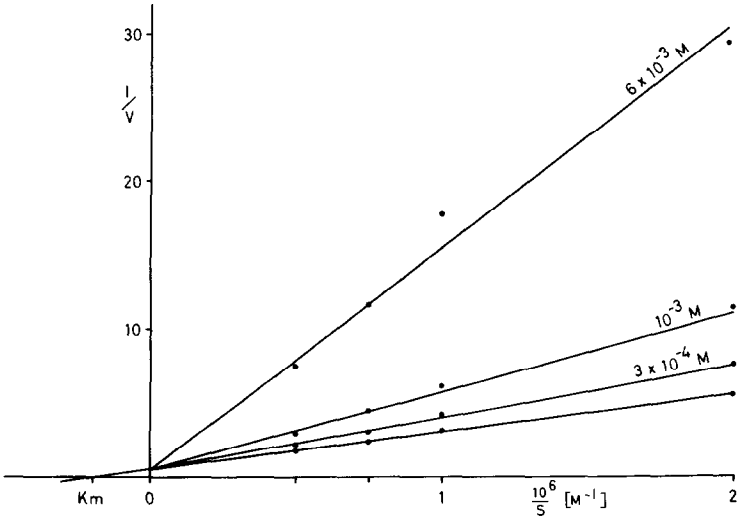

Fig. 4. Lineweaver–Burk plot of phosphodiesterase activity. Phosphodiesterase was assayed in homogenates of fat cells at substrate concentrations from 0·5 to 2·0 μM cyclic 3′,5′-AMP ($K_m = 5$ μM) in the absence and presence of the indicated concentrations of benzydamine. V = nmoles PO_4^{3-} liberated per 10^6 cells and 10 min. Each value represents the mean of 3 experiments performed in duplicate.

Table 2. Effect of benzydamine on cyclic 3',5'-AMP-dependent and independent protein kinase activity

	pmoles PO_4 transferred/mg protein × min	
	Without cAMP	5×10^{-8} M cAMP
Control	86 ± 4·2	593 ± 16·7
Benzydamine		
5×10^{-5} M	93 ± 4·0	621 ± 9·5
5×10^{-4} M	110 ± 2·4	646 ± 23·4
10^{-3} M	91 ± 5·6	603 ± 18·6

Incubations were performed at 25° for 30 min. Each value represents the mean ± S.E.M. of 2 experiments performed in duplicate.

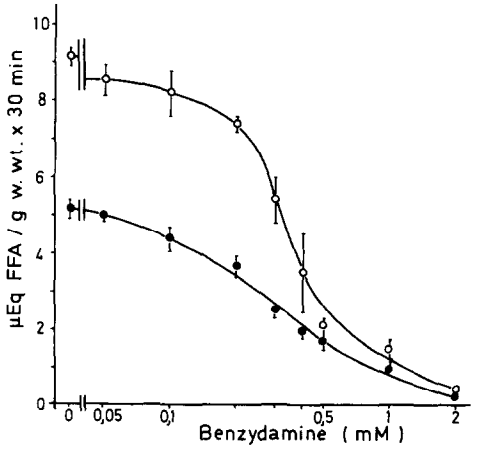

Fig. 5. Effect of benzydamine on the activity of hormone sensitive triglyceride lipase. Prestimulation with 1 µM NE (○——○) and without prestimulation (●——●). Incubations were performed for 30 min at 30°. Each value represents the mean ± S.E.M. of 5 experiments performed in duplicate.

meters and are effective in phosphorylating or activating lipase from adipose tissue [11].

Since the antilipolytic action of benzydamine cannot be explained by effects on other components of the lipolytic system of the fat cell, the direct interaction was studied with the hormone sensitive triglyceride lipase. Benzydamine causes a dose-dependent inhibition of lipase activity in adipose tissue homogenates. The dose–response curve closely correlates to the inhibitory effect on lipolysis in isolated fat cells, since 50% inhibition was obtained at almost identical concentrations (0·3–0·4 mM). The studies with tissue homogenates preclude effects of benzydamine on enzymes other than lipases, since activation of the lipases was achieved during a preincubation period in the absence of the drug. The 80% stimulation of lipase activity obtained during 5 min preincubation of fat pads with 1 µM NE prior to homogenization indicates that hormone-sensitive triglyceride lipase activity was measured under our experimental conditions, since this enzyme is the rate-limiting step for the splitting of triglycerides into FFA and glycerol by the consecutive action of triglyceride, diglyceride and monoglyceride lipase [12]. In regard to the degree of activation and time period for prestimulation our data are in close agreement with the findings of Crum *et al.* [13] and Huttunen *et al.* [9].

Interferences by benzydamine with ion translocations appear to be unlikely in adipose tissue homogenates, since the medium does not contain Mg^{2+} ions, necessary for the function of adenylate cyclase and protein kinase, and Ca^{2+} ions known to inhibit protein kinase activity and to activate phosphoprotein phosphatases [14, 15]. Furthermore, the latter enzyme is strongly inhibited by inorganic phosphate [14], present in our homogenization medium.

Benzydamine causes an increase in cyclic 3',5'-AMP levels following stimulation of the cells by NE. The accumulation of the cyclic nucleotide is also enhanced, when cyclic 3',5'-AMP levels are determined in the presence of NE plus theophylline. These findings are contrary to observations obtained with fibroblasts, in which the drug causes a dose-dependent decrease in cyclic 3',5'-AMP levels [1]. However, other compounds such as adenosine or prostaglandins are also known to have opposing influences on adenylate cyclases from different tissues [16, 17, 18].

The potentiating effect of benzydamine on the formation of cyclic 3',5'-AMP in fat cells stimulated by 10^{-6} M NE cannot be interpreted by its inhibition of cyclic nucleotide phosphodiesterase, since the drug does not prevent the secondary fall of cyclic 3',5'-AMP levels after 10 min. Moreover, phosphodiesterase activity of fibroblasts is also inhibited by the drug, although cyclic 3',5'-AMP levels are reduced in the cells. Furthermore, the effect of theophylline itself cannot only be linked to its inhibitory effect on phosphodiesterase, since Schwabe and Ebert [19] showed in their time-course studies on cyclic 3',5'-AMP in isolated fat cells that identical effects were obtained by theophylline and adenosine desaminase in regard to the extent and the maintenance of cyclic 3',5'-AMP levels in diluted fat cell suspensions as used in our study. These authors interpreted their findings as a competition between theophylline and adenosine for a regulatory site at the outer surface of the cell membrane. This adenosine receptor site is inhibitory for the adenylate cyclase in the isolated fat cell, but is far less effective in broken cell preparations or in fat cell ghosts [20]. Since benzydamine has a K_i for phosphodiesterase in the same order of magnitude as theophylline, it is unlikely that in contrast to theophylline benzydamine causes the observed elevation of cyclic 3',5'-AMP levels by affecting phosphodiesterase activity under these conditions.

Benzydamine shares the property of inhibiting stimulated lipolysis while further elevating cyclic 3',5'-AMP levels with a variety of compounds such

as tolbutamide and glibenclamide [21, 22], naphthoquinones [23, 24], tricyclic anti-depressants [25] and local anaesthetics [26]. The latter authors discussed the possibility, that local anaesthetics exert their antilipolytic action by influencing cation movements and especially Ca^{2+} translocations within the cell. In addition, several authors [27, 28, 29] showed that local anaesthetics affect Ca^{2+}-dependent processes in various tissues. Furthermore, Ca^{2+} ions appears to play an important role in the hormonal control of lipolysis [30, 31, 32] and other metabolic processes regulated by cyclic 3',5'-AMP (for review see Rasmussen, [33]).

Benzydamine possesses local anaesthetic activity as shown by corneal irritation tests [34]. Our results do not exclude the possibility that cation effects are responsible for the observed potentiation following stimulation of cyclic 3',5'-AMP levels by NE or NE plus theophylline. However, the inhibition of lipolysis appears to be due to direct inhibition of the activated hormone-sensitive triglyceride lipase by the drug. Therefore, the antilipolytic effect appears to be independent of any changes in ion concentrations.

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