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The Mechanism of the Lipolytic Action of Theophylline in Fat Cells

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MIYOSHI, Y., K. UCHIDA, E. TAKEDA-HARA, K. NAGAI AND H. OKUDA. *The mechanism of the lipolytic action of theophylline in fat cells.* PHARMAC. BIOCHEM. BEHAV. **14**(5) 701–706, 1981.—The lipolytic action of theophylline was examined using both intact fat cells and a fat globule system. Theophylline had similar lipolytic actions in both systems. However theophylline did not activate hormone-sensitive lipase in the fat globule system as measured with added Ediol. Pretreatment of the fat globules with phospholipase C suppressed theophylline-induced lipolysis, but phospholipase D had no effect. A theophylline-sensitive system was reconstituted from endogenous fat and a lipase fraction. Inhibitors of theophylline-induced lipolysis such as quinine and propranolol inhibited theophylline binding to artificial lipid micelles. Purine nucleosides such as adenosine, inosine and guanosine inhibited theophylline-induced lipolysis in the fat globule system. These results suggest that theophylline has a lipolytic action similar to that of adrenaline. Both share a lipolytic mechanism additional to that involving the activation of hormone sensitive lipase through the cyclic-AMP dependent protein kinase. Phospholipids play an important role in this additional mechanism.

Theophylline Lipolysis Phospholipid Adrenaline Phospholipase C Phospholipase D Lipase Fat cell Fat globule Nucleoside

IT has been shown that adrenaline, adrenocorticotropic hormone (ACTH) and theophylline have a lipolytic action in rat epididymal adipose tissue. Lipolysis induced by these agents is thought to be achieved through the activation of the hormone-sensitive lipase by the cyclic-AMP dependent protein kinase [1, 2, 5, 6, 12]. Thus theophylline is considered to act through its inhibitory action on the cyclic-AMP phosphodiesterase.

On the other hand Okuda and his colleagues [7, 10, 11, 19] have suggested that the lipolytic action of adrenaline and ACTH might not be due solely to the activation of the hormone-sensitive lipase. One suggestion that this might be so arose from the finding that a 48 hr fast did not increase the lipase activity of aqueous tissue extracts as assayed using externally added substrate emulsions and expressed on a per whole fat pad basis [11,16]. What then could account for the accelerated mobilization of fat from such tissue? To clarify this point Okuda *et al.* [7] prepared a cell free system which consisted of endogenous triglyceride substrate and lipase and responded to a lipolytic action of adrenaline. This was accomplished by subjecting isolated fat cells to hypoosmotic shock (5 mM Tris-HCl buffer, pH 7.4). The liberated fat globules were collected by gentle centrifugation and thoroughly washed. Addition of adrenaline to the resuspended globules nevertheless accelerated lipolysis without concomitant activation of protein kinase. This result indicated that the hormone could activate lipolysis by a mechanism independent of cyclic-AMP, possibly involving a modification of the substrate interface. This latter suggestion arose in part from the observation that the rate of hydrolysis of the fat globules by added pancreatic or bacterial lipases was also accelerated by adrenaline addition [19]. Recently Wise and Jungas [17] obtained additional evidence that adrenaline could activate lipolysis through a mechanism involving activation of the substrate.

In this paper we report that theophylline is also able to stimulate lipolysis in the washed fat globule system without activation of either the hormone-sensitive lipase or the protein kinase. Structural features essential for this stimulation by both methyl xanthines and catecholamines are described.

METHOD

Male Wistar rats housed in a room maintained at 25±1°C and 60% relative humidity were allowed free access to food and water. The room was illuminated for 12 hr a day starting at 7:00 a.m. Animals weighing 180–240 g were killed at

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around 3:00 p.m. and the epididymal adipose tissue was quickly excised. Fat cells were isolated by the procedure of Rodbell [13] and fat globules were prepared from the isolated cells as described previously [7]. Globules were washed three times in 5 mM Tris-Cl buffer, pH 7.4.

For the measurement of lipolytic activity whole fat cells were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 2.5% bovine serum albumin for 1 or 2 hr at 37°C in the presence of various chemicals. Washed fat globules were incubated similarly in 5 mM Tris-HCl, pH 7.4 containing 2.5% albumin. Free fatty acids (FFA) released during the incubation were extracted and titrated by Dole's method [4]. The activities of protein kinase and lipase were determined by the methods of Yamamura *et al.* [16] and of Rizack [12] using histone and Ediol (commercial preparation of coconut oil emulsion) as substrates respectively. The effects of phospholipase C and D were examined by incubating the fat globules for 10 min at 37°C with the enzymes.

Artificial lipid micelles were prepared by suspending 3 g of triolein and 300 mg of egg lecithin or phosphatidic acid or diglyceride in 30 ml of Krebs-Ringer phosphate buffer and sonicating for 3 min in an ice bath. Aliquots (2 ml) of this emulsion were centrifuged for 5 min at 3,500×g. After carefully removing the infranatant phase, the supernatant containing the lipid micelles was washed twice with fresh buffer. For the assay of theophylline binding, the lipid micelles were incubated with 0.5 μCi of ^{14}C-theophylline (2.7 mM) in a volume of 2 ml for 20 min at 37°C. The micelles were then washed twice with fresh buffer and their radioactivity determined using Patterson-Greene counting fluid (toluene:Triton X-100, 3:1).

All measurements were made in duplicate with the data expressed as means±standard deviations. Student's *t*-test was used for statistical analysis.

The source of chemicals and drugs was as follows: Theophylline, uric acid, imidozole and histamine, Wako Pure Chemical Co., Ltd.; adrenaline and isoxsupurine, Daiichi Pharmaceutical Co. Ltd.; noradrenaline, levodopa and salbutamol, Sankyo Co., Ltd.; l-isoproterenol, Nikken Chemicals Co., Ltd.; orciprenaline and etilefrine, C. H. Boehringer Sohn Co., Ltd.; ephedrine and spriphene, Hoechst Japan, Ltd.; dopamine, Kyowa Hakko Co., Ltd.; phenylephrine, Kowa Co., Ltd.; methoxamine, Nihon Shinyaku Co. Ltd.; telbutaline, Fujisawa Pharmaceutical Co., Ltd.; trimetoquinol, Tanabe Pharmaceutical Co., Ltd.; cyclic AMP (c-AMP), dibutyryl cyclic AMP (DBc-AMP) and adenosine, Boehringer Mannheim Co. Ltd.; caffeine and quinine, Hoei Yakko Co. Ltd.; diprophylline, Eizai Co., Ltd.; proxyphylline, Yoshitomi Pharmaceutical Co., Ltd.; propranolol, Sumitomo Chemical Co., Ltd.; Ediol (commercial emulsion of 50% coconut oil), Calbiochem, San Diego, California.

RESULTS

In Fig. 1 (upper curve) is shown the lipolytic response of intact fat cells to graded doses of theophylline. The values plotted in the figure are the increments in FFA production caused by the addition of theophylline. Note that when the washed fat globule preparation was incubated with theophylline a stimulation of lipolysis also occurred (lower curve), which was nearly identical to that seen with the intact cells.

To examine the mechanism of this theophylline effect in the fat globule system, an assay was performed for the lipase

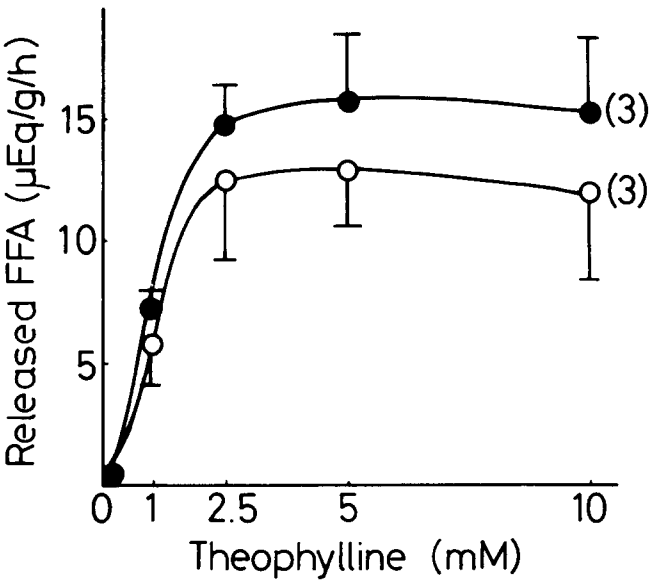

FIG. 1. Dose dependency of theophylline-induced lipolysis in intact fat cells and the fat globule preparation. Incubations were done for 1 hr at 37°C. Lipolytic activity was expressed as the increment in FFA release caused by the addition of theophylline. Results are averages of three experiments. Fat cell, ●——●; fat globule, ○——○.

activity associated with the globules. To accomplish this the globules were first incubated with and without theophylline for 30 min at 37°C. The globule suspensions were then homogenized using a Polytron homogenizer from Kinematica GmbH and portions of each homogenate were incubated with excess exogenous substrate in the form of Ediol for 1 hr. The results, summarized in the right panel of Fig. 2, revealed no change in lipase activity upon addition of theophylline. The left panel of Fig. 2 shows the lipolysis rate of the same fat globule preparation when only endogenous fat was present as substrate. Note that no homogenization was performed in this case. Theophylline caused more than a doubling in the release of FFA from the endogenous substrate. These results suggest that another mechanism besides the activation of the hormone-sensitive lipase might participate in the lipolytic action of theophylline in the fat globule system.

We have reported earlier that phospholipds are essential for the lipolytic action of adrenaline in the fat globule system [15]. Thus we examined whether this was also the case for theophylline. As shown in Fig. 3, pretreatment of the fat globules with high doses of crude phospholipase C (from *Clostridium perfringens*, Sigma Chemical Co.) completely blocked the lipolytic action of theophylline, while crude phospholipase D (from cabbage, Sigma Chemical Co.) was ineffective. When the phospholipase C was purified to homogeneity (using the method of Takeda-Hara, Nagai and Okuda, in preparation) as judged by polyacrylamide gel electrophoresis it was effective at much smaller concentrations. These findings suggest that phospholipid, especially the phosphoryl-choline group, is also essential for the lipolytic action of theophylline in the fat globule system.

Saito *et al.* [14] were also able to reconstitute an

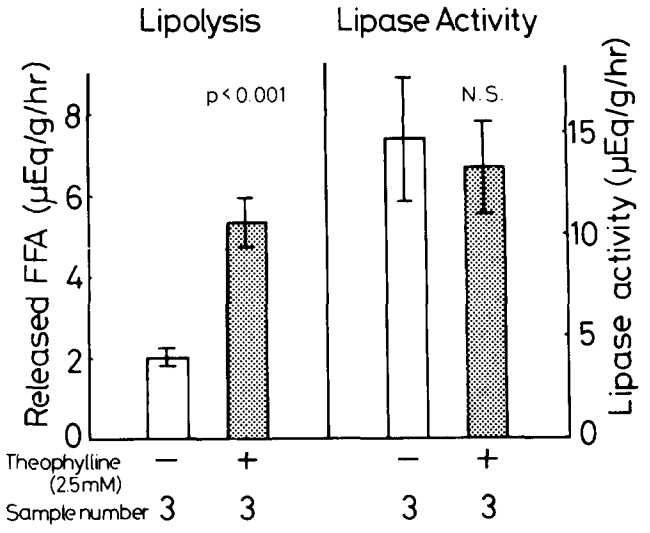

FIG. 2. Effect of theophylline on lipolysis and hormone-sensitive lipase activity in fat globule preparation. Left: Fat globules were incubated for 1 hr in the absence or presence of theophylline (2.5 mM). Right: After 30 min incubation of fat globules at 37°C with or without theophylline, globules were homogenized with a Polytron homogenizer. Then 0.5 ml of the homogenate (equivalent to the globules from 25 mg of adipose tissue) was incubated with 0.05 ml of 1:5 diluted solution of Ediol in 5% bovine serum albumin and 0.45 ml of 0.06 M phosphate buffer (pH 7.4) for 1 hr at 37°C. Lipase activity was expressed as FFA released per g tissue weight per hour. N.S.: not significant.

adrenaline-sensitive cell-free lipolytic system consisting of lipase-free lipid micelles and a fat-free lipase fraction. We have attempted similar experiments with theophylline. The fat globule preparation was treated witn 0.025% (w/v) Triton X-100 in 5 mM Tris-HCl buffer, pH 7.4, and then washed with the same buffer without the detergent by centrifugation at 300 rpm for 30 sec. The lipid micelles, now devoid of lipase activity, were incubated with a fat-free lipase solution, namely, the infranatant obtained by centrifuging a 20% (w/v) homogenate of rat epididymal adipose tissue in Krebs-Ringer phosphate buffer at 8,000 rpm for 30 min at 4°C. The results are summarized in Table 1 and indicate that theophylline was also effective in the reconstituted system. Pretreatment of the lipase-depleted lipid micelles with phospholipase C again blocked the response to theophylline.

We chose next to examine the binding of ^{14}C-theophylline to artificial lipid micelles. In this case we used micelles prepared from triolein and egg lecithin since Okuda *et al.* [9] had shown previously that such micelles were also effective in reconstituting a cell-free lipolytic system responsive to theophylline. As shown in Table 2, the addition of 10% by weight of egg lecithin to the triolein micelles markedly increased theophylline binding. Addition of phosphatidic acid was also effective but diglyceride had only a small effect. These results complement the earlier findings with phospholipase treatment in suggesting the importance of phospholipid in the binding of theophylline to the lipid micelles. We also observed that two agents effective in blocking lipolysis in intact fat cells or in the fat globule system, quinine and propranolol, were able to reduce the binding of theophylline to the triolein-lecithin micelles. The importance of this effect of quinine and of propranolol to their antilipolytic action in intact cells is unknown.

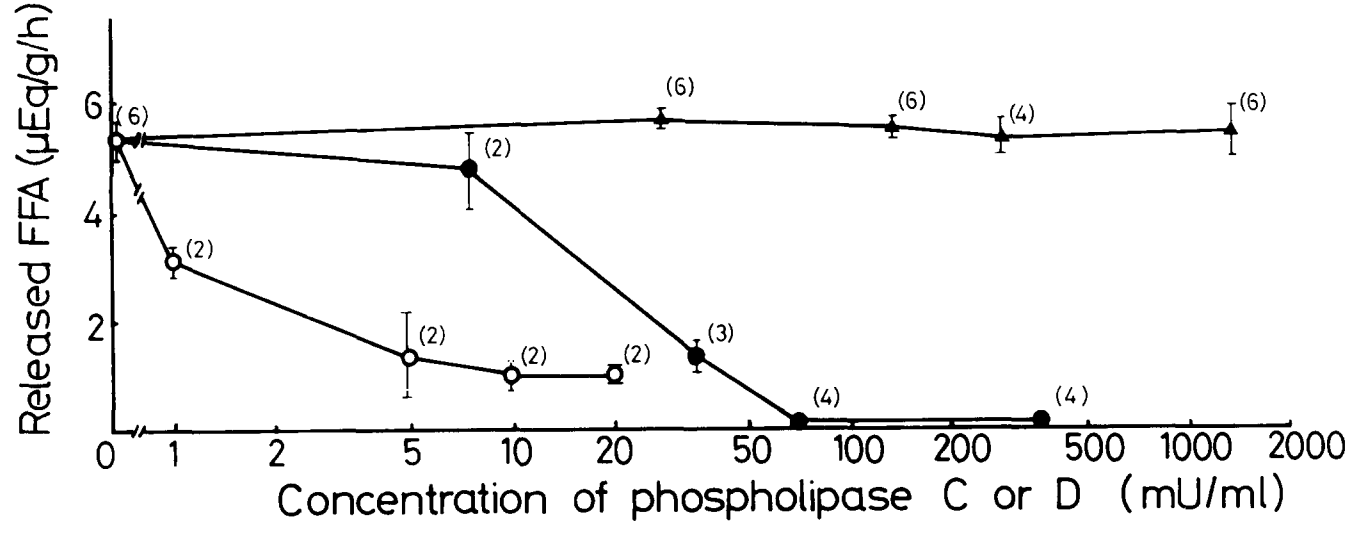

FIG. 3. Effect of pretreatment of fat globules with phospholipase C or D on theophylline-induced lipolysis. Fat globules (equivalent to 0.2 g of adipose tissue) were pretreated with phospholipase C or D at the concentrations indicated for 10 min at 37°C, then incubated for 1 hr at 37°C with or without 2.7 mM theophylline. The increase in FFA release caused by theophylline addition is shown. Number of experiments shown in parentheses. Crude phospholipase C, ●——●; purified phospholipase C, ○——○; crude phospholipase D, ▲——▲.

TABLE 1

RECONSTITUTION OF A THEOPHYLLINE-SENSITIVE LIPOLYTIC SYSTEM

Additions		Released FFA (μEq/g/hr)	
Infranatant Solution	Lipid Micelles	Theophylline	
		−	+
−	+	0.0	0.0
+	−	0.5	0.5
+	+	2.6	10.0
+	Phospholipase C	2.0	2.1

Triton-treated lipid micelles equivalent to 200 mg tissue were suspended in 0.5 ml KRP buffer containing 5% bovine serum albumin and incubated for 1 hr at 37°C with 0.5 ml aliquots of the infranatant solution described in the text. When present the theophylline concentration was 2.7 mM. For treatment with phospholipase C the washed lipid micelles were incubated for 10 min at 37°C in Krebs-Ringer phosphate buffer containing 500 μg/ml of the crude enzyme. The treated lipid micelles were washed twice by centrifugation with the same buffer and resuspended.

Another antilipolytic substance thought to be physiologically important is adenosine as first reported by Dole [3]. We therefore examined the effects of a series of nucleosides on the lipolytic action of theophylline, both in intact fat cells and in the fat globule system. As shown in Table 3, adenosine and inosine were quite effective inhibitors while guanosine had a lesser effect. Pyrimidine nucleosides were without effect.

We next sought to clarify the structural requirements for lipolysis activation in the fat globule system by examining a series of purine derivatives. Lipolysis in intact fat cells was also measured for comparison. The results given in Table 4 reveal that theophylline and dibutyryl cyclic-AMP were very effective in both systems while cyclic-AMP itself had little effect. Diprophylline was also active in the fat globule system.

Finally, we conducted a more extensive examination of the structural requirements for activation of lipolysis by catecholamines and related compounds in both systems. The structural formulae of these chemicals were shown in Fig. 4.

Structure :

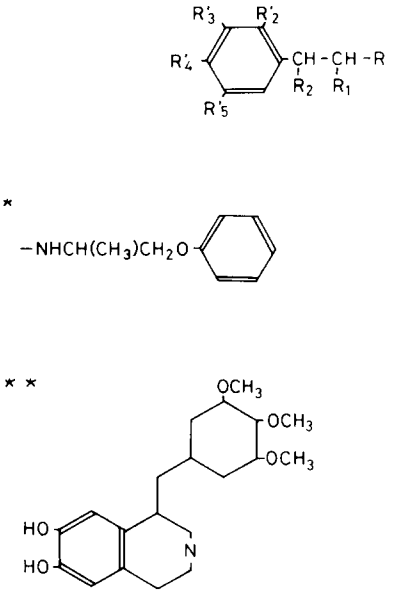

FIG. 4. Structural formulae of catecholamines and related compounds. R, R_1, R_2, R_2', R_3', R_4', R_5', * and ** are corresponded to those in Table 5.

From the results shown in Table 5 we conclude that activity in the two systems is always comparable and requires the catechol nucleus and amino group on the side chain. The presence of methoxy substituents on the aromatic ring (methoxamine) leads to complete loss of activity. The necessary features of the ethylamino side chain remain to be fully established.

In some of these experiments the cyclic-AMP dependent protein kinase activity of both fat cell extracts and the fat globule system was measured. These data are also shown in Table 5 as the activity ratio, i.e., activity without added cyclic-AMP as a percentage of activity with excess cyclic-AMP. In the fat cells adrenaline increased this activity ratio,

TABLE 2

^{14}C-THEOPHYLLINE BINDING TO ARTIFICIAL LIPID MICELLES

Composition of Lipid Micelles	Additions		Bound Radioactivity (cpm)
triolein alone	theophylline	(2.7 mM)	1050 ± 174 (3)
triolein+egg lecithin	theophylline	(2.7 mM)	6696 ± 783 (3)
triolein+phosphatidic acid	theophylline	(2.7 mM)	4330 ± 417 (3)
triolein+diglyceride	theophylline	(2.7 mM)	2466 ± 27 (3)
triolein+egg lecithin	theophylline	(2.7 mM)	3134 ± 216 (3)
	quinine	(5.0 mM)	
triolein+egg lecithin	theophylline	(2.7 mM)	3378 ± 450 (3)
	propranolol	(3.0 mM)	

Conditions of the experiment are described in the text.

TABLE 3

EFFECT OF NUCLEOSIDES ON THEOPHYLLINE-INDUCED LIPOLYSIS IN INTACT FAT CELLS AND FAT GLOBULE PREPARATIONS

Nucleosides (10^{-4} M)	Fat cells % Change	Fat globules % Change
Adenosine	−40	−33
Inosine	−33	−25.7
Guanosine	−17	−15
Xanthosine	−2	−7.6
Cytidine	−0.5	−2.9
Uridine	−3	+12.3
Thymidine	+17	0

Fat cells or fat globules equivalent to 0.2 g tissue were incubated with theophylline, 2.5 mM and nucleosides as indicated for 2 hr at 37°C. Control values without nucleosides were 9.3 ± 2.4 for fat cells and 5.5 ± 0.6 for fat globules expressed as μEq/g/hr.

Data in the table are expressed relative to these values.

TABLE 4

LIPOLYTIC ACTIONS OF METHYLXANTHINES AND RELATED COMPOUNDS

Chemicals	(mM)	Fat cells (μEq/g/2 hr)	Fat globules (μEq/g/2 hr)
Theophylline	5.0	13.0	9.3
Cyclic-AMP	1.0	0.3	0.1
DiButyryl-cyclic-AMP	1.0	8.5	11.8
Caffeine	1.8	5.0	—
Diprophylline	1.8	4.6	4.3
Proxyphylline	1.8	4.0	—
Adenine	1.8	0.0	—
Uric acid	3.3	0.0	—
Imidazole	1.8	0.0	—
Histamine	1.8	0.0	—
Adenosine	1.8	0.0	—

Values shown are increments over control values.

as expected from the elevation in cellular cyclic-AMP levels caused by this agent. In the fat globule system, however, no such effect was observed, presumably because of the absence of plasma membranes and thus of adenylate cyclase from this system. These data further indicate that the lipolytic action of adrenaline in the fat globule preparation is not mediated by cyclic-AMP. Other catecholamines tested behaved similarly except for ephedrine which at high concentrations activated the protein kinase but had little effect on lipolysis.

DISCUSSION

Even though it requires a concentration roughly 1,000 times higher, theophylline exerts a lipolytic effect similar to that of adrenaline in both the intact fat cell and the fat globule system. Pretreatment with purified phospholipase C or the addition of purine nucleosides such as adenosine block or decrease the lipolytic action of theophylline and adrenaline in the fat globule system. A reconstituted system consisting of lipase-free lipid micelles and fat-free lipase solutions retain their sensitivity to both theophylline and adrenaline, and

TABLE 5

LIPOLYTIC ACTIONS OF CATECHOLAMINES AND RELATED COMPOUNDS

Chemicals	R'_5	R'_4	R'_3	R'_2	R_2	R_1	R	(mM)	Release of fatty acids (μEq/g/2 hr) Fat cells	Release of fatty acids (μEq/g/2 hr) Fat globules	Protein kinase activity ratio $(\frac{-\text{c-AMP}}{+\text{c-AMP}} \times 100)$ Fat cells	Protein kinase activity ratio $(\frac{-\text{c-AMP}}{+\text{c-AMP}} \times 100)$ Fat globules
None								0	0.1	0.0	38.0	49.5
Adrenaline	-OH	-OH	H	H	-OH	H	$-NHCH_3$	4.5×10^{-3}	16.0	14.0	55.4	44.5
Noradrenaline	-OH	-OH	H	H	-OH	H	$-NH_2$	9.5×10^{-3}	10.3	10.0	—	40.0
l-Isoproterenol	-OH	-OH	H	H	-OH	H	$-NH(CH_3)_2$	3.6×10^{-2}	13.6	10.1	—	58.3
Orciprenaline	-OH	H	-OH	H	-OH	H	$-NHCH(CH_3)_2$	1.6×10^{-1}	11.0	10.4	—	44.2
Ephedrine	H	H	H	H	-OH	$-CH_3$	$-NHCH_3$	1.8	1.3	—	—	81.9
Dopamine	-OH	-OH	H	H	H	H	$-NH_2$	1.8	15.3	7.3	—	—
Levodopa	-OH	-OH	H	H	H	$-NH_2$	-COOH	1.2	0.0	—	—	—
Etilefrine	H	H	-OH	H	-OH	H	$-NHCH_2CH_3$	1.8	2.2	2.4	—	—
Phenylephrine	H	H	-OH	H	-OH	H	$-NHCH_3$	2.2	3.8	1.8	—	—
Spriphene	H	-OH	H	H	-OH	$-CH_3$	$-NHCH_3$	4.2	11.8	8.3	—	—
Methoxamine	$-OCH_3$	H	H	$-OCH_3$	-OH	$-NH_2$	$-CH_3$	1.8	0.0	—	—	—
Isoxsupurine	H	-OH	H	H	-OH	$-CH_3$	*	2.5	2.1	0.1	—	—
Salbutamol	H	-OH	$-CH_2OH$	H	-OH	H	$-NHC(CH_3)_3$	1.8	8.5	10.8	—	—
Telbutaline	-OH	H	-OH	H	-OH	H	$NHC(CH_3)_3$	6×10^{-2}	5.0	6.5	—	58.5
Trimetoquinol			**					4.2×10^{-2}	13.8	11.0	—	50.3

0.5 ml of intact fat cells suspended in Krebs-Ringer phosphate buffer (pH 7.4) or fat globules suspended in 5 mM Tris-Cl buffer (pH 7.4) (approximately 200 mg per tube) were incubated for 2 hr with 0.5 ml of Krebs-Ringer phosphate buffer containing 5% bovine serum albumin and 0.1 ml of catecholamine solution. The structural formulae are shown in Fig. 4.

again phospholipase C blocks the activation process. It seems most important to point out that these lipolytic actions in the fat globule system do not depend on the activation of hormone-sensitive lipase through cyclic-AMP dependent protein kinase since neither protein kinase nor lipase activity changed with either theophylline or adrenaline addition to fat globule systems. Thus at least in this system another mechanism besides the activation of lipase must participate in the lipolytic action of theophylline and adrenaline. Concerning this mechanism, Wise and Jungas [17] also reported that endogenous substrate activation was elicited by the incubation with adrenaline in rat adipose tissue, and they also proposed another lipolytic mechanism besides the activation of hormone-sensitive lipase through cyclic-AMP dependent protein kinase. From this finding and the data summarized above, we think the mechanism of the lipolytic action of adrenaline and theophylline in fat cells is as follows: adrenaline and theophylline penetrate the cell membrane and combine with phospholipid present in lipid micelles in the fat globule. This combination increases the affinity of the lipase toward its substrate, the lipid micelles, thus stimulating lipolysis [8]. Whether this is the case or not must await further evidence.

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