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EFFECTS OF SODIUM SALICYLATE AND ACETYLSALICYLIC ACID ON THE LIPOLYTIC SYSTEM OF FAT CELLS

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Abstract—Sodium salicylate and acetylsalicylic acid both cause a dose-dependent inhibition of lipolysis in isolated fat cells stimulated by norepinephrine or dibutyryl-cyclic 3′,5′-AMP. The lipolytic system is affected at several stages. (1) Salicylate reduces ATP-levels in the cells in the same dose-range as inhibition of lipolysis, while acetylsalicylic acid is without effect. (2) Accumulation of cyclic 3′,5′-AMP is decreased by both compounds. (3) Phosphodiesterase is inhibited by both compounds. (4) Binding of cyclic 3′,5′-AMP to cyclic 3′,5′-AMP-dependent protein kinase is reduced in presence of both compounds, salicylate being more effective than acetylsalicylic acid. Inhibition of lipolysis stimulated by dibutyryl-cyclic 3′,5′-AMP indicate that the observed reduction of binding between protein kinase and cyclic 3′,5′-AMP may be essential for the antilipolytic effect of both compounds.

THE ANTILIPOLYTIC effect of sodium salicylate (SA) was first described by Carlson and Östman[1] in normal and diabetic patients. Since lipolysis in fat cells is regulated via cyclic 3′,5′-AMP,[2,3] the antilipolytic effect of SA may be effected by an influence on one or more components of the adenyl cyclase system. Carlson and Östman[1] showed that SA also decreased lipolysis in fat tissue *in vitro* not stimulated by hormones. Stone *et al.*[4] investigated the antilipolytic effect of SA in isolated fat cells stimulated by hormones such as norepinephrine (NE), ACTH, glucagon and dexamethasone plus growth hormone. In all cases SA inhibited lipolysis. Since this effect was also observed when theophylline or dibutyryl-cyclic 3′,5′-AMP was present in the incubation medium, the results indicated an action of SA beyond the formation of cyclic 3′,5′-AMP.

The present study is an attempt to determine the site of action of SA and acetylsalicylic acid (ASA) in the lipolytic system of the fat cell. A preliminary account of some of this work has been presented.[5]

MATERIALS AND METHODS

Materials were obtained from following sources: bovine albumin, fraction V, powder (Armour Pharmaceutical Co.); bacterial collagenase and lyophilized firefly

* Some of this work is part of the Doctoral thesis of Joachim Sohn.

lanterns (Worthington Biochemical Corp.); l-norepinephrine bitartrate (Fluka AG); theophylline (Merck, Darmstadt); ATP, cyclic 3′,5′-AMP and N^6O^2-dibutyryl-cyclic 3′,5′-AMP (Boehringer, Mannheim); ^{32}P-cyclic 3′,5′-AMP (420 Ci/mM) (Radiochemical Centre, Amersham); ^{3}H-adenine (6 Ci/mM) and ^{3}H-cyclic 3′,5′-AMP (24·1 Ci/mM) (New England Nuclear).

Male Wistar rats (160–220 g) were used in all experiments. Fat cell preparations were performed according to Rodbell[6] in Krebs–Ringer phosphate buffer (pH 7·4) containing 1 % albumin.

Lipolysis. Fat cell preparation (usually 3–4 $\times$ 10^6 cells/ml) were diluted with Krebs–Ringer phosphate buffer (pH 7·4) containing 5 % (w/v) albumin and half the recommended Ca^{2+} concentration to give 2–4 $\times$ 10^4 cells/ml. Assay of lipolytic activity in presence and absence of SA and ASA was performed as described earlier.[7] Lipolysis was expressed as micromoles glycerol released per 10^6 cells per hour at 37°.

Phosphodiesterase assay. Phosphodiesterase activity was assayed in homogenates of fat cells at 37°, using low concentrations (4 $\times$ 10^{-6} M and less) of substrate and measuring the release of $^{32}P_i$ from ^{32}P-cyclic 3′,5′-AMP according to the method of Schönhöfer *et al.*[8] Activities were expressed as nanomoles P_i liberated per 10^6 cells in 10 min at 37°.

Conversion of prelabelled nucleotides to cyclic 3′,5′-AMP. ^{3}H-Adenine incorporation into cyclic 3′,5′-AMP was measured according to the method of Kuo and Derenzo[9] as modified by Schönhöfer and Skidmore.[10] Prelabelled fat cells were diluted with Krebs–Ringer phosphate buffer (pH 7·4) containing 5 % (w/v) albumin to give 2–4 $\times$ 10^4 cells/ml and about 1–4 $\times$ 10^5 counts/min/ml total radioactivity. One ml samples were incubated with 10^{-6} M NE and 5 $\times$ 10^{-4} M theophylline in the presence of varying concentrations of SA and ASA at 37° for 10 min and assayed for ^{3}H-cyclic 3′,5′-AMP formed. The results were expressed as percentage of total radioactivity within the cells converted into cyclic 3′,5′-AMP during incubation.

ATP-levels. ATP-levels in fat cells were determined according to the method of Kalbhen and Koch[11] during incubations performed under identical conditions as measurement of the conversion of labelled nucleotides to cyclic 3′,5′-AMP. After incubation, 0·2 ml of the incubation mixtures were added to 1·0 ml boiling 0·05 M glycine buffer (pH 11·0). Half a millilitre of this solution was used for ATP determination by the luciferin–luciferase method. Results were expressed as nanomoles ATP per 10^6 cells.

Binding of cyclic 3′,5′-AMP to cyclic 3′,5′-AMP-dependent protein kinase. Protein kinase was prepared from beef diaphragm muscle according to the method of Miyamoto *et al.*[12] Inhibition of binding of cyclic 3′,5′-AMP to protein kinase was assayed according to the method of Gilman.[13] Incubations were performed with 1 and 5 pmoles ^{3}H-cyclic 3′,5′-AMP in presence of varying concentrations of SA and ASA in a total volume of 0·3 ml containing 2·5 μg protein kinase at 0° for 90 min. Inhibition of binding was expressed as percentage of the amount of binding of the standard (usually 20–25 per cent of the added cyclic 3′,5′-AMP).

To determine the type of inhibition of binding between cyclic 3′,5′-AMP and protein kinase, the amount of protein kinase was reduced to 0·8 μg to achieve linearity of the binding reaction for more than 30 min. Under these conditions incubations were performed in the presence of 0·1–1·0 pmoles ^{3}H-cyclic 3′,5′-AMP and varying concentrations of SA and ASA at 0° for 20 min.

RESULTS

In isolated fat cells SA and ASA both cause a dose-dependent inhibition of maximal lipolysis elicited either by 10^{-6} M Ne or 3×10^{-3} M dibutyryl-cyclic 3′,5′-AMP (Figs. 1 and 2), whereas unstimulated lipolysis was unaffected (Table 1). In the same

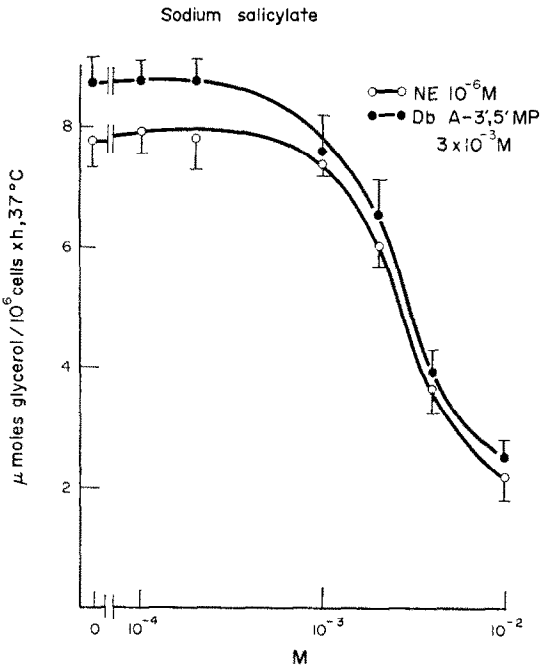

FIG. 1. Effect of sodium salicylate on lipolysis. Dose–response curve of SA on lipolysis of isolated fat cells stimulated maximally by 10^{-6} M Ne (○——○) and 3×10^{-3} M dibutyryl-cyclic 3′,5′-AMP (●——●). Values represent the mean ±S.E.M. of three experiments each performed in duplicate. Lipolysis expressed as micromoles glycerol released per 10^6 cells per hour.

dose-range conversion of prelabelled nucleotides into cyclic 3′,5′-AMP is reduced by both compounds, even though the ATP-levels, determined under identical conditions, are not affected by ASA (Figs. 3 and 4). This indicates a direct effect of ASA on adenyl cyclase in intact cells. SA reduces the ATP-levels in cells parallel to the inhibition of conversion of prelabelled nucleotides into cyclic 3′,5′-AMP, thus preventing an analysis of the action of SA on adenyl cyclase.

Since inhibition of lipolysis may be caused by activation of phosphodiesterase the influence of SA and ASA on the activity of this enzyme was investigated. Both compounds showed an inhibitory effect on phosphodiesterase activity (Figs. 5 and 6). The inhibition was competitive in nature with K_i values of 8×10^{-3} M for SA and $3{\cdot}3 \times 10^{-3}$ M for ASA.

Contrary to results obtained by measuring the accumulation of cyclic 3′,5′-AMP following stimulation of prelabelled cells by 10^{-6} M NE in the presence of 5×10^{-4} M theophylline and SA or ASA, assay of cyclic 3′,5′-AMP levels by the Gilman method showed no decrease but a slight increase in cyclic 3′,5′-AMP levels with increasing concentrations of SA and ASA. Since SA and ASA were present in the cyclic 3′,5′-

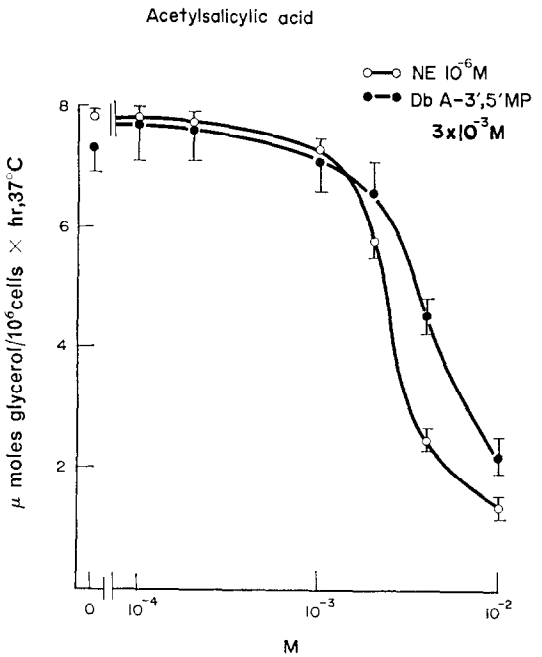

FIG. 2. Effect of acetylsalicylic acid on lipolysis. Dose–response curve of ASA on lipolysis of isolated fat cells stimulated maximally by 10^{-6} M NE (○——○) or 3×10^{-3} M dibutyryl-cyclic 3',5'-AMP (●——●). Values represent the mean ±S.E.M. of three experiments each performed in duplicate. Lipolysis expressed as micromoles glycerol released per 10^6 cells per hour.

TABLE 1. EFFECT OF SALICYLATE AND ACETYLSALICYLIC ACID ON UNSTIMULATED LIPOLYSIS

	Lipolysis (μmoles glycerol/10^6 cells × hr) inhibited by:	
	Salicylate	Acetylsalicylic acid
No additions	0·38 ± 0·11	0·52 ± 0·04
10^{-2} M	0·38 ± 0·16	0·58 ± 0·17
6×10^{-3} M	0·34 ± 0·06	0·61 ± 0·17
10^{-3} M	0·31 ± 0·06	0·60 ± 0·23
10^{-4} M	0·32 ± 0·04	0·59 ± 0·15

Incubations performed as described in Materials and Methods. Lipolysis expressed as micromoles of glycerol released per 10^6 cells per hour. Each value represents the mean ±S.E.M. of three experiments performed in duplicate.

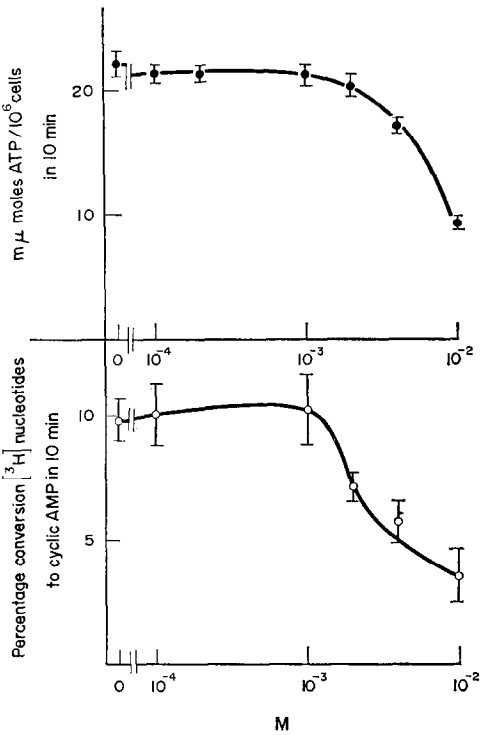

FIG. 3. Effect of sodium salicylate on ATP-levels and cyclic 3′,5′-AMP accumulation in fat cells. Upper section shows dose–response curve of SA on ATP-levels in fat cells after 10 min incubation. ATP-levels expressed as nanomoles ATP/10^6 cells. Lower section shows dose–response curve of SA on the accumulation of cyclic 3′,5′-AMP in prelabelled cells. Results expressed as percentage of total radioactivity within the cells converted into cyclic 3′,5′-AMP after 10 min incubation. In all cases 10^{-6} M NE and 5×10^{-4} M theophylline were present for stimulation of adenyl cyclase. Each value represents the mean ±S.E.M. of three experiments each performed in duplicate or triplicate.

AMP fraction after separation of the nucleotides by Dowex AG 50 columns, the effect of both compounds was tested on the binding reaction between cyclic 3′,5′-AMP and protein kinase. In the presence of $3{\cdot}3 \times 10^{-9}$ and $16{\cdot}5 \times 10^{-9}$ M ^{3}H-cyclic 3′,5′-AMP both compounds reduced dose-dependently the amount of binding between cyclic 3′,5′-AMP and protein kinase when measurement was performed under the "steady state" conditions of the Gilman assay (Table 2). No changes in the binding reaction were found when corrections were made according to ionic strength of the incubation mixture, either by use of equimolar concentrations of NaCl or other organic acids. Analysis of the type of inhibition showed competitive pattern for SA ($K_i = 3 \times 10^{-3}$ M) (Fig. 7).

DISCUSSION

In agreement with observations by Stone *et al.*[4] our results show that SA and ASA both inhibit NE-stimulated lipolysis in isolated fat cells at concentrations achieved in therapeutic treatment, since daily doses of 5–8 g ASA result in blood levels of $1–3 \times 10^{-3}$ M. Contrary to findings by Carlson and Östman[1] with fat tissue, unstimulated lipolysis was not affected in isolated fat cells.

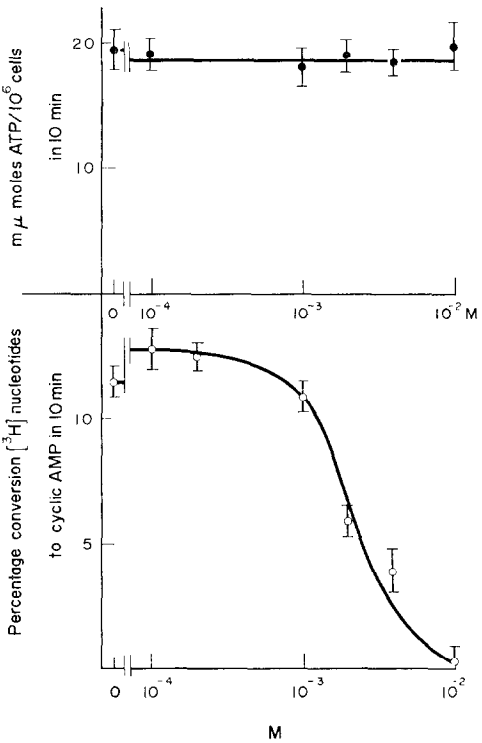

FIG. 4. Effect of acetylsalicylic acid on ATP-levels and cyclic 3′,5′-AMP accumulation in fat cells Presentation of results are identical with Fig. 3.

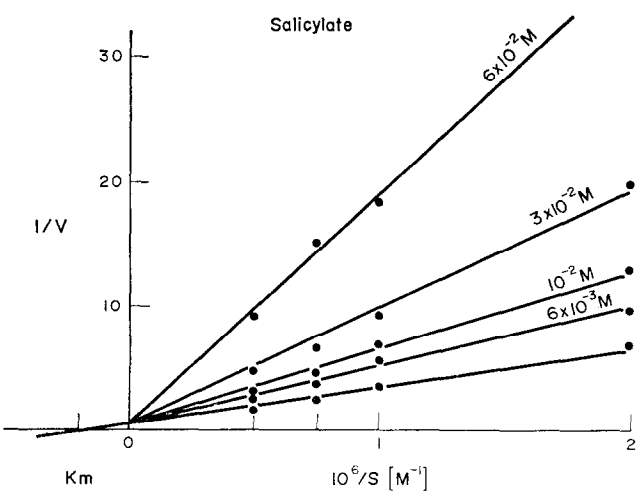

FIG. 5. Effect of salicylate on phosphodiesterase activity in fat cell homogenates. Incubations performed as described in Materials and Methods in the presence of 5×10^{-7} to 2×10^{-6} M ^{32}P-cyclic 3′,5′-AMP as substrate and 6×10^{-3} to 6×10^{-2} M SA. Phosphodiesterase activity expressed as nmoles $^{32}P_i$ liberated per 10^6 cells per min. Each value represents the mean of two experiments performed in duplicate or triplicate. $K_m = 5 \times 10^{-6}$ M, V: [nmoles $^{32}P_i/10^6$ cells $\times$ min].

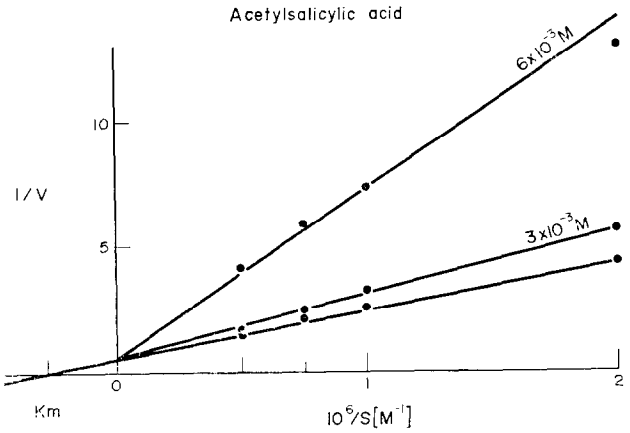

FIG. 6. Effect of acetylsalicylic acid on phosphodiesterase activity in fat cell homogenates. Presentation of results are identical with Fig. 5.

TABLE 2. EFFECT OF SODIUM SALICYLATE AND ACETYLSALICYLIC ACID ON THE BINDING OF CYCLIC 3′,5′-AMP TO CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE

	Inhibition of binding (%) (Substrate ^{3}H-cyclic 3′,5′-AMP)	
	$3{\cdot}3 \times 10^{-9}$ M	$1{\cdot}65 \times 10^{-8}$ M
Control (counts/min bound)	1717 ± 18	1557 ± 145
Sodium salicylate		
10^{-2} M	70·9 ± 1·7	49·2 ± 2·9
6×10^{-3} M	41·7 ± 3·9	34·0 ± 4·0
3×10^{-3} M	25·7 ± 2·0	29·1 ± 5·5
10^{-3} M	13·1 ± 3·0	19·5 ± 3·8
3×10^{-4} M	2·7 ± 5·4	13·0 ± 5·6
10^{-4} M	(±5·0)	(±4·3)
Acetylsalicylic acid		
10^{-2} M	23·6 ± 2·3	26·6 ± 2·8
6×10^{-3} M	20·4 ± 3·8	23·3 ± 0·8
3×10^{-3} M	11·4 ± 5·6	15·5 ± 3·1
10^{-3} M	8·6 ± 6·8	3·5 ± 2·5
3×10^{-4} M	(±7·2)	(±5·0)
10^{-4} M	(±6·1)	(±7·6)

Incubations performed with 1 and 5 pmoles ^{3}H-cyclic 3′,5′-AMP as substrate, 1·5–2·5 μg protein kinase and SA or ASA for 90 min at 0° as described in Materials and Methods. Results expressed as percentage inhibition of binding of cyclic 3′,5′-AMP. Values represent the mean ±S.E.M. of three experiments each performed in duplicate or triplicate.

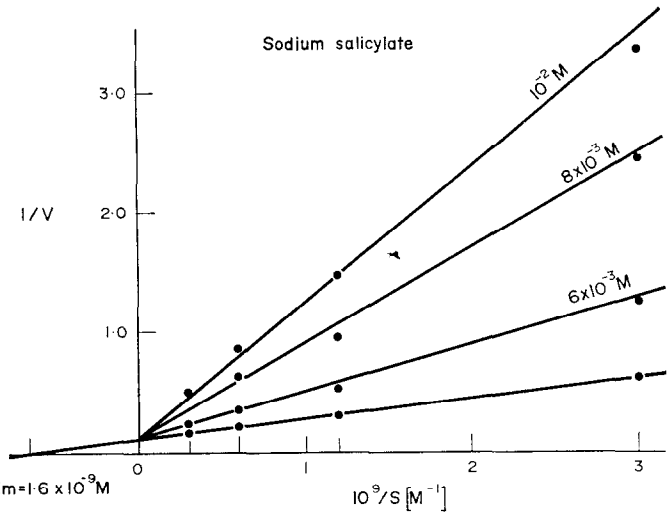

FIG. 7. Effect of salicylate on cyclic 3′,5′-AMP-dependent protein kinase. Competitive inhibition of binding of cyclic 3′,5′-AMP to cyclic 3′,5′-AMP dependent protein kinase. Incubations performed in presence of $3{\cdot}3 \times 10^{-10}$ to $3{\cdot}3 \times 10^{-9}$ M ^{3}H-cyclic 3′,5′-AMP, 0·8 μg protein kinase and 6×10^{-3} to 6×10^{-2} M SA for 20 min at 0° as described in Materials and Methods. Binding was expressed as counts/min ^{3}H-cyclic 3′,5′-AMP bound/microgram protein kinase × min. Each value represents the mean of two experiments performed in duplicate. $K_m = 1{\cdot}6 \times 10^{-9}$ M, V: [counts/min cyclic 3′,5′-AMP bound per milligram protein kinase per min] × 10^3.

The mechanism by which both compounds influence the lipolytic system appears to result from several factors. The NE-stimulated accumulation of cyclic 3′,5′-AMP is reduced in the same dose range in which inhibition of lipolysis occurs. This decrease in conversion of ATP into cyclic 3′,5′-AMP appears not to be solely secondary to a decrease of ATP-levels by uncoupling of oxidative phosphorylation,[14] since ASA depresses the accumulation of cyclic 3′,5′-AMP without affecting the ATP content of the cells.

Inhibition of lipolysis can be caused by activation of phosphodiesterase which by lowering intracellular cyclic 3′,5′-AMP levels may reduce the lipolytic response. Our results show, however, a competitive inhibition of phosphodiesterase by both compounds. Furthermore, the inhibitor constants of $K_i = 8 \times 10^{-3}$ M for SA and $K_i = 3{\cdot}3 \times 10^{-3}$ M for ASA are in a similar dose range as that of theophylline ($K_i = 0{\cdot}87 \times 10^{-3}$ M) determined under identical conditions.[8] These results indicate that SA and ASA have a similar inhibitory effect on phosphodiesterase activity as theophylline, even though lipolysis and accumulation of cyclic 3′,5′-AMP are decreased. They support the findings by Schwabe *et al.*[15] and Dalton *et al.*[16] that the potentiating effect of methylxanthines on lipolysis and cyclic 3′,5′-AMP levels may be caused by other mechanisms than inhibition of phosphodiesterase.

Both compounds inhibit also lipolysis stimulated by dibutyryl-cyclic 3′,5′-AMP. Therefore, the essential metabolic lesion in lipolysis caused by both compounds appeared to be beyond the formation of cyclic 3′,5′-AMP by adenyl cyclase. Our experiments show that SA and ASA impair the ability of cyclic 3′,5′-AMP to bind to cyclic 3′,5′-AMP-dependent protein kinase at concentrations at which lipolysis is inhibited. The inhibition is of competitive type. Since binding of cyclic 3′,5′-AMP to

the "regulatory site" of the protein kinase is responsible for the dissociation of the active enzyme from its inactive complex,[17] inhibition of binding between cyclic 3′,5′-AMP and inactive protein kinase may result in an impairment of the function of this enzyme and consequently in an inhibition of phosphorylation of the inactive triglyceride lipase of the cell. Our results indicate that this impairment of function of the cyclic 3′,5′-AMP-dependent protein kinase may cause the observed inhibition of lipolysis by SA and ASA.

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