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EFFECTS OF SODIUM SALICYLATE AND ACETYLSALICYLIC ACID ON CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE

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Abstract—Sodium salicylate and to a lesser extent acetylsalicylic acid both inhibit the activity of cyclic 3′,5′-AMP-dependent protein kinase measured by the phosphorylation of histone. The competitive type of inhibition is only seen in the presence of cyclic 3′,5′-AMP. In the absence of cyclic 3′,5′-AMP the unstimulated activity of protein kinase is increased. The impairment of function of protein kinase correlates with the previously reported inhibition of binding of cyclic 3′,5′-AMP to cyclic 3′,5′-AMP-dependent protein kinase and indicates that the action of sodium salicylate may involve the "regulatory site" of the protein kinase.

PREVIOUS studies from this[1] and other laboratories[2-3] have shown that both sodium salicylate (SA) and acetylsalicylic acid (ASA) inhibit lipolysis in fat cells stimulated by hormones such as norepinephrine and by dibutyryl-cyclic 3′,5′-AMP. Attempting to determine the metabolic lesion caused by SA and ASA in the lipolytic system of the fat cell we were able to show that both compounds impaired the ability of cyclic 3′,5′-AMP to bind to the cyclic 3′,5′-AMP-dependent protein kinase (PK). The present study investigates the impairment of function of cyclic 3′,5′-AMP-dependent PK caused by SA and ASA.

MATERIALS AND METHODS

Materials were obtained from following sources: Histone, Schuchardt, München; [γ-^{32}P]-labelled ATP (20·3 Ci/mmole), Amersham–Buchler, Braunschweig; cyclic 3′,5′-AMP, Boehringer, Mannheim. All other chemicals used were reagent grade from the usual commercial sources.

Protein kinase was prepared from bovine diaphragm muscle using the simplified method described by Gilman[4]. The PK eluted with 300 mM potassium phosphate (pH 7·0) from a DEAE-cellulose column (Whatmann DE 52, microgranular) was used in all experiments. PK activity was determined according to Miyamoto *et al.*[5] with modifications, in a total volume of 200 μl containing: 50 mM glycerol phosphate (pH 6·5), 10 mM $MgCl_2$, 0·3 mM ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetra-acetic acid, 10 μM [γ-^{32}P]-ATP (sp. act. 5–50 mCi/mM), 0·5 mg histone and cyclic 3′,5′-AMP and PK as indicated in text. Incubations, started by addition of PK diluted in 20 μl 5 mM potassium phosphate buffer (pH 6·0), were performed at 25° for 30 min. The reaction was stopped by addition of 1·0 ml 10% (w/v) trichloroacetic acid followed by 0·1 ml 1·2% (w/v) albumin as carrier. The samples were kept on ice for 5 min and centrifuged for 2 min at 12 000 *g*. The supernatant was discarded, the precipitate redissolved in 0·2 ml 1 N NaOH and reprecipitated by 1·0 ml 10% (w/v) trichloroacetic acid. The washing procedure was repeated twice. After dissolving the

final precipitate in 0·2 ml 1 N NaOH the plastic incubation tubes (Eppendorf No. 3180) were placed in dry glass scintillation vials and counted in a liquid scintillation counter by use of the Tscherenkov-effect.

Results were expressed as pmoles $^{32}PO_4$ bound/mg PK/min. By this procedure the non-bound $^{32}PO_4$ was always less than 2 per cent of the experimental values and mostly about 0·1 per cent. Therefore, this blank was not subtracted from the experimental values.

RESULTS

The protein kinase preparation, used in our experiments, was characterized by following data.

Phosphorylation of histone. 200 pmoles $^{32}PO_4$/mg PK × min. in the absence of cyclic 3′,5′-AMP, 1800 pmoles $^{32}PO_4$/mg PK × min in the presence of 5×10^{-6} M cyclic 3′,5′-AMP.

Sensitivity to cyclic 3′,5′-AMP. Stimulation was observed at concentrations of cyclic 3′,5′-AMP above 10^{-9} M and was maximal above 10^{-7} M ($K_m = 2{\cdot}69 \times 10^{-8}$ M).

Binding capacity for cyclic 3′,5′-*AMP.* 60–80 pmoles cyclic 3′,5′-AMP/mg PK (in absence of inhibitor protein) when determined under the conditions of the protein binding assay described by Gilman.[4]

When PK was incubated with histone, [γ-^{32}P]-ATP and varying concentrations of cyclic 3′,5′-AMP, SA caused a competitive inhibition of PK activity (Fig. 1). The apparent inhibition constant was $K_i = 3 \times 10^{-3}$ M for SA. When PK was incubated

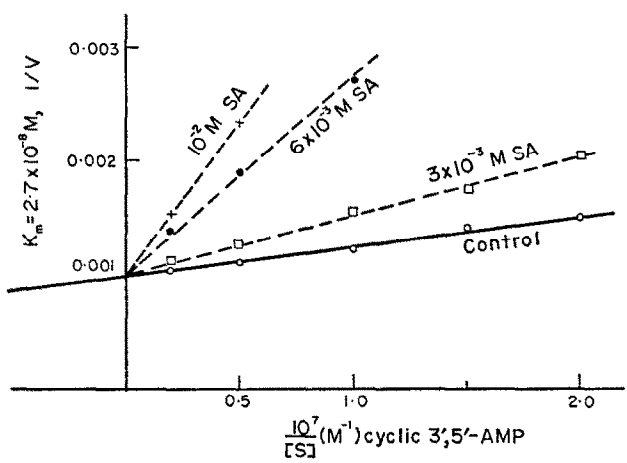

FIG. 1. Effect of sodium salicylate on cyclic 3′,5′-AMP-dependent protein kinase activity. Incubations were performed as described in Materials and Methods with 1·64 μg protein kinase in the presence of varying concentrations of cyclic 3′,5′-AMP. Protein kinase activity is expressed as picomoles $^{32}PO_4$ bound/milligram PK × minute. Each value represents the mean of two experiments performed in duplicate. V (pmoles $^{32}PO_4$/mg PK × min).

with histone and a submaximal concentration of cyclic 3′,5′-AMP (5×10^{-8} M) in the presence of varying concentrations of ATP, SA again caused a competitive inhibition of PK activity with an apparent $K_i = 7{\cdot}5 \times 10^{-4}$ M (Fig. 2).

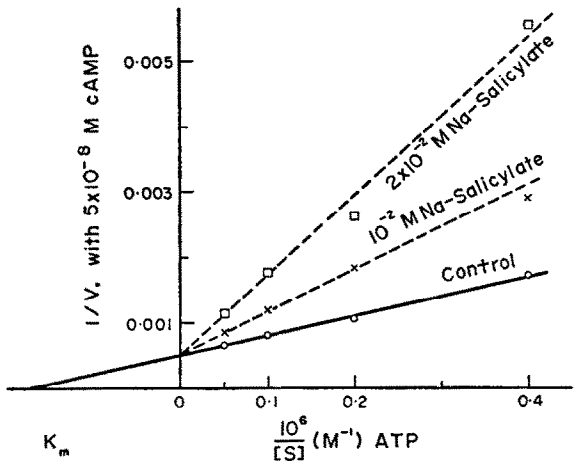

FIG. 2. Effect of sodium salicylate on cyclic 3′,5′-AMP-dependent protein kinase in the presence of 5×10^{-8} M cyclic 3′,5′-AMP. Incubations were performed as described in Materials and Methods with 1·64 μg protein kinase in the presence of varying concentrations of ATP. Protein kinase activity is expressed as picomoles $^{32}PO_4$ bound/mg PK $\times$ min. Each value represents the mean of two experiments performed in duplicate. V (pmoles $^{32}PO_4$/mg PK $\times$ min).

In the absence of cyclic 3′,5′-AMP, however, SA caused a dose-dependent stimulation of PK activity (Fig. 3). This stimulation results in an increase of the V_{max} from 178 pmoles $^{32}PO_4$/mg PK $\times$ min to 455 pmoles $^{32}PO_4$/mg PK $\times$ min in the presence of 10^{-2} M SA without change of the K_m.

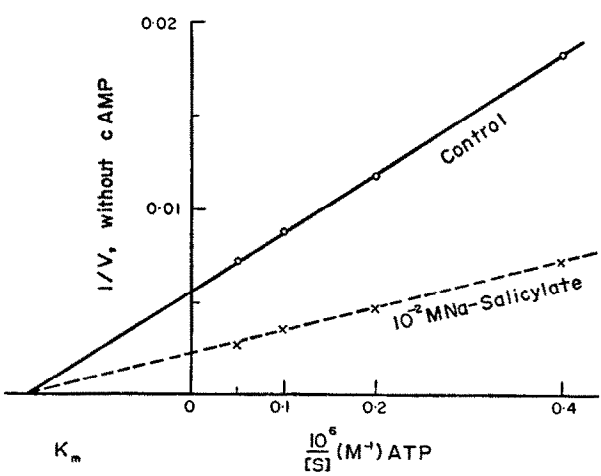

FIG. 3. Effect of sodium salicylate on cyclic 3′,5′-AMP-dependent protein kinase in the absence of cyclic 3′,5′-AMP. Incubations were performed as described in Materials and Methods with 8·2 μg protein kinase in the presence of varying concentrations of ATP. Protein kinase activity is expressed as pmoles $^{32}PO_4$ bound/mg PK $\times$ min. Each value represents the mean of two experiments performed in duplicate. V (pmoles $^{32}PO_4$/mg PK $\times$ min).

Table 1 shows the dose-range in which the inhibitory and stimulatory effect of SA on PK activity was observed. ASA exhibited a similar inhibitory effect on PK in the presence of cyclic 3′,5′-AMP, but was less effective in stimulating PK activity in the absence of the cyclic nucleotide (Table 2).

TABLE 1. EFFECT OF SODIUM SALICYLATE ON CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE IN THE PRESENCE AND ABSENCE OF CYCLIC 3′,5′-AMP

	pmoles $^{32}PO_4$ bound/mg PK × min	
	With cyclic 3′,5′-AMP (5×10^{-8} M)	Without cyclic 3′,5′-AMP
Control	1392 ± 20·9	173 ± 7·6
Sodium salicylate		
10^{-4} M		206 ± 4·7
3×10^{-4} M		237 ± 12·2
6×10^{-4} M		253 ± 4·6
10^{-3} M	1372 ± 64·8	330 ± 9·1
3×10^{-3} M	1266 ± 13·9	392 ± 12·7
6×10^{-3} M	1100 ± 29·5	450 ± 8·9
10^{-2} M	936 ± 22·5	472 ± 10·0
2×10^{-2} M	618 ± 29·9	475 ± 12·6

Inhibition of cyclic 3′,5′-AMP-dependent protein kinase in the presence of 5×10^{-8} M cyclic 3′,5′-AMP and stimulation of the enzyme in the absence of cyclic 3′,5′-AMP. Incubations were performed as described in Materials and Methods with 1·64 μg protein kinase in the presence and 8·2 μg in the absence of cyclic 3′,5′-AMP. Each value represents the mean ± S.E.M. of two or three experiments performed in dublicate or triplicate.

TABLE 2. EFFECT OF ACETYLSALICYLIC ACID ON CYCLIC 3′,5′-AMP-DEPENDENT PROTEIN KINASE IN THE PRESENCE AND ABSENCE OF CYCLIC 3′,5′-AMP

	pmoles $^{32}PO_4$ bound/mg PK × min	
	With cyclic 3′,5′-AMP (5×10^{-8} M)	Without cyclic 3′,5′-AMP
Control	1392 ± 20·9	173 ± 7·6
Acetylsalicyclic acid		
3×10^{-4} M		177 ± 9·5
10^{-3} M	1383 ± 27·7	200 ± 4·6
3×10^{-3} M	1246 ± 22·0	204 ± 8·2
6×10^{-3} M	1136 ± 19·6	244 ± 10·6

Inhibition of cyclic 3′,5′-AMP-dependent protein kinase in the presence of 5×10^{-8} M cyclic 3′,5′-AMP and stimulation in the absence of the stimulant. Incubations were performed as described in Materials and Methods with 1·64 μg protein kinase in the presence and 8·2 μg in the absence of cyclic 3′,5′-AMP. Each value represents the mean ± S.E.M. of two or three experiments performed in duplicate or triplicate.

DISCUSSION

In our previous study on the antilipolytic effect of SA and ASA[1] we found that both compounds influenced the ability of cyclic 3′,5′-AMP-dependent PK to bind cyclic 3′,5′-AMP. Our data showed that SA inhibited the binding between the enzyme and the cyclic nucleotide competively with an apparent $K_i = 3 \times 10^{-3}$ M. Binding of cyclic 3′,5′-AMP to inactive PK activates the enzyme by dissociation of the catalytic subunit from the "regulatory" or cyclic 3′,5′-AMP binding subunit.[6–10] It was interesting therefore, to test the phosphorylating activity of the enzyme in presence of SA and ASA, since the competitive inhibition of binding between the cyclic nucleotide and PK would not necessarily affect the activity of the enzyme if SA and ASA also caused a dissociation of the catalytic subunit from the inactive enzyme.

Our results show, however, that SA and ASA both inhibit the phosphorylation of histone by cyclic 3′,5′-AMP-dependent PK when the enzyme is activated by the cyclic nucleotide. In the presence of submaximal concentrations of cyclic 3′,5′-AMP approximately equal to tissue levels[11], the inhibition is more prominent than at maximal concentrations of the stimulant (5×10^{-7} M). As in our studies on the binding of cyclic 3′,5′-AMP to PK,[1] the inhibition of the phosphorylation of histone, caused by SA, is competitive in nature, giving an identical apparent $K_i = 3 \times 10^{-3}$ M both for inhibition of binding and of phosphorylation. These results indicate that the observed inhibition of binding between the cyclic nucleotide and the enzyme by SA may be related to the decreased activity of the enzyme in phosphorylating histone and that SA may cause inhibition of PK by competing with the cyclic nucleotide at the "regulatory" site of the enzyme.

These findings should, however, be interpreted with care, since SA also affects the activation and function of the enzyme. In the absence of cyclic 3′,5′-AMP, SA showed an "intrinsic activity" on PK when tested against varying concentrations of ATP. This resulted in an increase of the V_{max} of phosphorylation without change of the K_m and may indicate an influence on the regulatory site releasing catalytic subunits.

In the presence of submaximal concentrations of cyclic 3′,5′-AMP, however, SA exhibited again a competitive inhibition of histone phosphorylation when plotted against varying concentrations of ATP, thus apparently competing also with the nucleotide at the catalytic site. It was not possible to clarify this additional influence in our test system, since in our experiments only the combined inactive form of the enzyme was available.

Since in fat cells the main function of PK is the phosphorylation of the triglyceride lipase as target enzyme of the lipolytic system, the observed inhibition of PK may explain the antilipolytic effect of both compounds. This assumption is supported by results with lipolysis stimulated by dibutyryl-cyclic 3′,5′-AMP acting presumably like intracellular cyclic 3′,5′-AMP after penetration of the cell wall.[12] Dibutyryl-cyclic 3′,5′-AMP stimulated lipolysis is inhibited by SA and ASA as well as hormone stimulated lipolysis,[1-2] indicating that the essential metabolic lesion caused by both compounds was beyond the formation of cyclic 3′,5′-AMP within the cells.

The observed stimulation of PK activity by SA and ASA in the absence of cyclic 3′,5′-AMP correlates with the lack of effect of both compounds on unstimulated lipolysis as reported in our previous study.[1] Moreover, we were able to show that compounds such as paraoxon acting directly on triglyceride lipase also inhibited

unstimulated lipolysis.[13-14] An increase of unstimulated lipolysis, however, was not observed in our experiments. This finding is to be expected from our data, as SA exhibits its "intrinsic" activity only in absence of cyclic 3',5'-AMP. In fat cells, low concentrations of the cyclic nucleotide are always present, even when cells are not treated by any stimulating agent.

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REFERENCES

1. P. S. SCHÖNHÖFER, J. SOHN, H. D. PETERS and V. DINNENDAHL, *Biochem. Pharmac.* **22,** 629 (1973).
2. D. B. STONE, J. D. BROWN and A. A. STEELE, *Metabolism* **18,** 620 (1969).
3. A. CARLSON and J. ÖSTMAN, *Metabolism* **10,** 781 (1961).
4. A. G. GILMAN, *Proc. natn. Acad. Sci. U.S.A.* **67,** 305 (1970).
5. E. MIYAMOTO, J. F. KUO and P. GREENGARD, *J. biol. Chem.* **244,** 6395 (1969).
6. C. O. BROSTROM, J. D. CORBIN, C. A. KING and E. G. KREBS, *Proc. natn. Acad. Sci. U.S.A.* **68,** 2444 (1971).
7. G. N. GILL and L. D. GARREN, *Biochem. biophys. Res. Commun.* **39,** 335 (1970).
8. E. M. REIMANN, C. O. BROSTROM, J. D. CORBIN, C. A. KING and E. G. KREBS, *Biochem. biophys. Res. Commun.* **42,** 187 (1971).
9. M. TAO, M. L. SALAS and F. LIPMANN, *Proc. natn. Acad. Sci. U.S.A.* **67,** 408 (1970).
10. L. J. CHEN and D. A. WALSH, *Biochemistry* **10,** 3614 (1971).
11. G. A. ROBISON, R. W. BUTCHER and E. W. SUTHERLAND, *Cyclic AMP*, pp. 285–316. Academic Press, London (1971).
12. H. HILZ, E. KAUKEL and W. TARNOWSKI, *Int. Conf. Physiol. Pharmac. of Cyclic AMP*, Milan (1971), p. 576, Raven Press, New York (1972).
13. H. D. PETERS, P. SELHORST, V. DINNENDAHL, K. U. HELM and P. S. SCHÖNHÖFER, *Archs Toxic.* **30,** 139 (1973).
14. V. DINNENDAHL, H. D. PETERS, K. U. HELM and P. S. SCHÖNHÖFER, *Archs Toxic.* **30,** 175 (1973).