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EFFECT OF FLUORIDE ON LIVER PHOSPHORYLASE PHOSPHATASE

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SUMMARY

Perfusion of dog liver *in situ* with NaF or incubation of liver extracts with NaF resulted in inactivation of phosphorylase phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17). *In vitro* this process was found to be dependent on time, temperature and concentration of fluoride. Reactivation could be obtained with ATP in the presence of Mg^{2+}. These interconversions were of a stable character. This inactivation of phosphorylase phosphatase is unrelated to the well known inhibition of the enzyme by NaF. The inactive forms isolated from livers perfused with either NaF or NaCl displayed identical properties, as judged from their activation by ATP and Mg^{2+}.

INTRODUCTION

In avian liver[1] and in dog liver perfused with NaF[2] phosphorylase phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17) exists in an active and inactive form and activation can be obtained by incubation of the enzyme preparation with ATP in the presence of Mg^{2+}. However, we have observed that perfusion of dog liver *in situ* with NaF instead of NaCl resulted in a lower phosphorylase phosphatase activity and an enhanced activation by ATP and Mg^{2+}. Therefore it was of interest to study the effect of NaF on phosphorylase phosphatase *in vitro*, and to compare the inactive forms obtained after NaF or NaCl perfusion.

EXPERIMENTAL PROCEDURE

Enzymes

Active phosphorylase (α-1,4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1) was the third ethanol fraction obtained from dog liver as described by SUTHERLAND AND WOSILAIT[3] and was dialyzed against glass distilled water.

Phosphorylase phosphatase was isolated from dog liver by a method described previously[2] except that the livers were perfused with either 0.15 M NaCl or 0.2 M NaF and the final preparations were dialyzed against glass distilled water.

Activation of phosphorylase phosphatase

Complete activation was obtained by incubation at 30° for 10 min in the presence of 4 mM ATP, 5 mM $MgSO_4$, 0.1 mM EDTA and 40 mM Tris (pH 7.4). The activated as well as the nonactivated samples were dialyzed against 10 mM Tris (pH 7.4) and diluted several hundredfold before the assay.

The amount of inactive phosphorylase phosphatase was calculated by subtracting the activity of controls (nonactivated) from the total activity obtained after incubation in the presence of ATP and Mg^{2+}.

Determination of phosphorylase phosphatase activity

Phosphorylase phosphatase activity[2] was measured at 30° by following the inactivation of the purified phosphorylase (0.3 unit/ml). In the assay mixture 5 mM caffeine, 44 mM Tris (pH 7.4), and 0.1 mM EDTA were included when using homogenates; and 0.5 mM caffeine, 8 mM Tris (pH 7.4) and 0.01 mM EDTA, in the determination of the activity of the purified phosphorylase phosphatase. One unit of phosphorylase phosphatase inactivates 1 I.U. of phosphorylase per min under these experimental conditions.

Phosphorylase activity was measured at 30° by incubating 0.1 ml of the enzyme with 0.1 ml of a solution containing 0.1 M α-D-glucose 1-phosphate, 2% glycogen, and 0.2 M NaF, adjusted to pH 6.1. The reaction was stopped by adding 0.5 ml 10% trichloroacetic acid, and inorganic phosphate was then determined[4].

RESULTS

Effect of NaF in the perfusion medium on phosphorylase phosphatase activity

From the data shown in Table I one can see that the livers, perfused with NaF,

TABLE I

THE EFFECT OF NaF PERFUSION ON PHOSPHORYLASE PHOSPHATASE MEASURED IN LIVER HOMOGENATES

Homogenates were made in 1 vol. 10 mM Tris (pH 7.4). The liver of dog 31-3 was first perfused with NaCl and subsequently with NaF, while in the dogs 7-4 and 21-4 part of the liver was removed before the NaF perfusion was started. In the experiment with dog 12-5 one lobe was perfused separately with NaCl while the remaining liver was perfused with NaF. The results are expressed as units of phosphorylase phosphatase per g liver.

Dog	*NaF perfusion*			*Control*		
	Active phosphatase	*Inactive phosphatase*	$\left(\frac{Inactive}{Active}\right)$	*Active phosphatase*	*Inactive phosphatase*	$\left(\frac{Inactive}{Active}\right)$
6-5	9.2	24.6	2.67	—	—	—
16-6	19.8	16.9	0.85	—	—	—
7-7				16.3	2.3	0.14
7-8	4.6	9.6	2.09	—	—	—
31-3	10.2	8.7	0.85	40.2	5.8	0.14
1-4				14.1	9.8	0.70
7-4	5.2	9.9	1.90	20.0	3.3	0.17
21-4	11.2	6.7	0.60	21.4	4.7	0.22
12-5	20.5	9.0	0.44	40.4	6.2	0.15
Mean (S.E.)	11.5 (2.4)	12.2 (2.4)	1.34 (0.33)*	25.4 (4.8)	5.3 (1.1)	0.25 (0.09)*

* $P = 0.0046$ (Wilcoxon's test).

contained more inactive than active phosphorylase phosphatase than the NaCl perfused controls. As a consequence the ratio of inactive to active phosphatase was significantly increased. These phenomena are not due to residual NaF, since they persisted after several hundredfold dilution or after precipitation with $(NH_4)_2SO_4$ and dialysis. A slight reactivation of inactive phosphatase was obtained with 5 mM $MgSO_4$ after NaF or NaCl perfusion (not shown). The total activity of phosphorylase phosphatase was slightly lower after perfusion with NaF; this indicated a decreased stability of the enzyme after NaF perfusion.

Effect of NaF in dialyzed liver extracts

The inactivation of phosphorylase phosphatase by NaF (Fig. 1) was dependent on time and temperature. Preincubation in the presence of 0.2 M NaCl at 30° had no effect on phosphorylase phosphatase activity (not shown). Since the effect of NaF at higher temperature easily resulted in an irreversible inactivation, the inactivation using NaF was carried out at 20°.

No effect on phosphorylase phosphatase activity was observed when these extracts were incubated for 24 min at 30° in the presence of 1 mM EDTA. Higher concentrations of EDTA caused an irreversible inactivation.

The results shown in Fig. 2 indicate that the inactivation of the previously

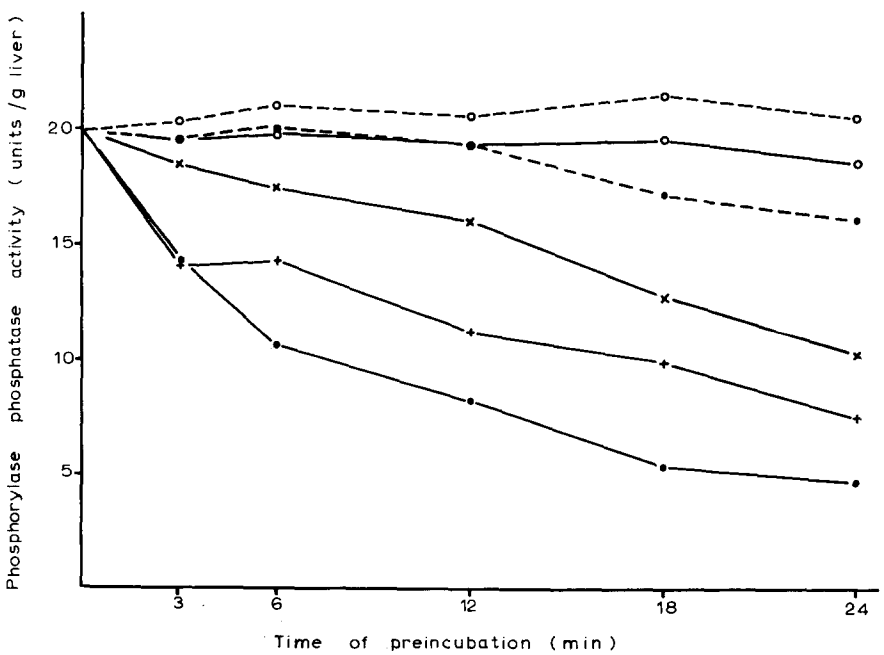

Fig. 1. Effect of NaF on phosphorylase phosphatase in dialyzed liver extracts. After perfusion with NaCl the liver was homogenized with 1 vol. of 10 mM Tris (pH 7.4), and then centrifuged at 8000 × *g* for 10 min. The supernatant was dialyzed against 10 mM Tris (pH 7.4). The preparation was then diluted 10-fold and incubated without any addition at 0° (○---○), at 37° (●---●), or in the presence of 40 mM NaF at 0° (○—○), 20° (×—×), 30° (+—+) and 37° (●—●). The reaction was stopped by diluting 15-fold with the same Tris buffer, and active phosphorylase phosphatase was assayed.

activated phosphorylase phosphatase is dependent on the concentration of the F^-. An almost complete reactivation was obtained by incubation with ATP in the presence of Mg^{2+}.

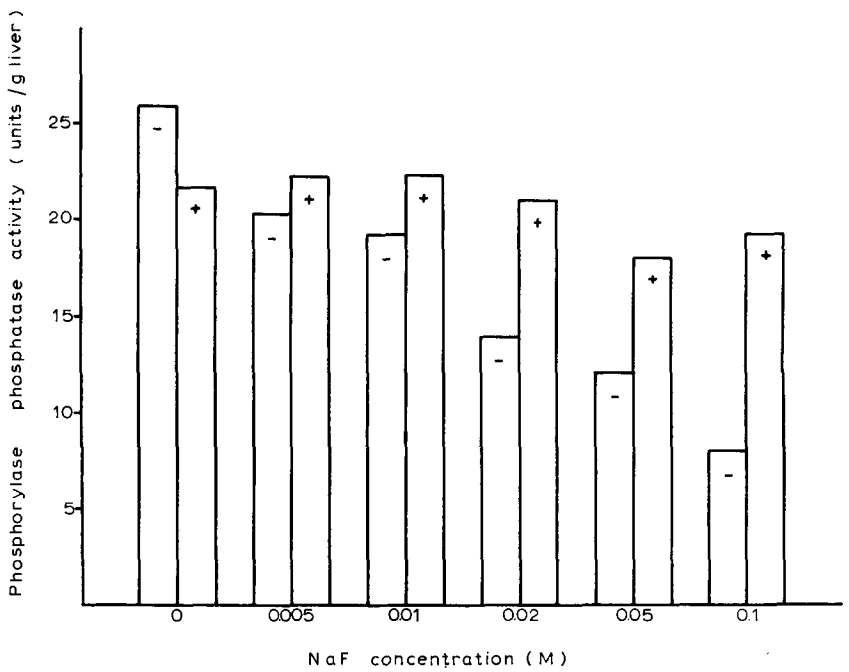

Fig. 2. Inactivation *in vitro* of phosphorylase phosphatase by different concentrations NaF, and reactivation by ATP and Mg^{2+}. A liver ectract was prepared as described in Fig. 1. After activation of the phosphatase, the preparation was dialyzed and diluted 10-fold with 10 mM Tris (pH 7.4). It was then incubated at 20° for 30 min in the presence of the stated concentrations of NaF. The incubated samples were either kept at 0° (−) or reactivated (+) by the procedure described in EXPERIMENTAL PROCEDURE. All the fractions were subsequently dialyzed against Tris buffer prior to the assay of phosphorylase phosphatase.

The reversible inactivation caused by NaF ocurred only in dialyzed preparations since high concentrations (0.2 M at 30°) were necessary in untreated homogenates.

When an homogenate was incubated with NaF (Fig. 2) in the presence of 4 mM ATP and 5 mM $MgSO_4$ activation always occurred. However, the phosphorylase phosphatase reaction was nearly completely inhibited by 20 mM NaF in the presence or absence of 4 mM ATP and 5 mM $MgSO_4$.

Properties of the purified inactive liver phosphorylase phosphatase after perfusion with NaF or NaCl

Some properties of the inactive phosphorylase phosphatase were investigated with the purified enzyme fractions since these preparations are rich in the inactive enzyme and free of phosphorylase kinase (ATP: phosphorylase phosphotransferase, EC 2.7.1.38)[2]. The study of the activation of the purified phosphorylase phosphatase preparations obtained from NaF perfused livers could be performed because the

control assay showed that NaF had induced a substantial inactivation (see Table I). Reversible inactivation of the phosphatase did not occur during purification, since previously activated homogenate in the presence of ATP and $MgSO_4$ showed practically no activation.

As shown in Table II the purified phosphorylase phosphatase obtained after fluoride perfusion showed a lower activity with a higher degree of activation than the enzyme obtained from control livers. These preparations were less stable than after NaCl perfusion. No difference could be observed in the time course of activation and in both preparations the activation was not affected by cyclic AMP (adenosine 3′:5′-monophosphate).

TABLE II

THE EFFECT OF NaF PERFUSION ON THE PURIFIED PHOSPHORYLASE PHOSPHATASE

Perfusion techniques are given in Table I. The results are expressed as units of phosphorylase phosphatase per ml of the preparation.

Dog	*NaF perfusion*			*Control*		
	Active phosphatase	*Inactive phosphatase*	$\left(\frac{Inactive}{Active}\right)$	*Active phosphatase*	*Inactive phosphatase*	$\left(\frac{Inactive}{Active}\right)$
7-4	1.0	8.1	8.1	5.6	8.2	1.5
21-4	0.6	12.3	20.5	6.1	10.5	1.7
12-5	2.6	22.8	8.8	5.8	10.6	1.8

The effects of ATP and $MgSO_4$ on phosphorylase phosphatase activity were similar for both preparations (Table III): low concentrations of ATP in the presence of Mg^{2+} were efficient for enzyme activation; incubation with 5 mM $MgSO_4$ resulted in only a slight activation of the phosphorylase phosphatase. With ATP alone inactivation was observed.

TABLE III

EFFECT OF DIFFERENT CONCENTRATIONS OF ATP AND/OR Mg^{2+} ON PURIFIED PHOSPHORYLASE PHOSPHATASE

The incubations were carried out at 30° for 30 min when ATP alone was added, and for 15 min in all other conditions. The reaction was stopped by suitable dilution with cold 10 mM Tris (pH 7.4), and the results are expressed as units of phosphorylase phosphatase per ml of the preparation.

Additions	*Preparation*	
	NaF	*NaCl*
None	0.2	7.1
$MgSO_4$, 5 mM	0.5	8.7
1 mM	0.2	6.9
ATP, 4 mM; $MgSO_4$, 5 mM	3.4	19.0
0.8 mM; $MgSO_4$, 1 mM	3.3	17.6
0.4 mM; $MgSO_4$, 0.5 mM	2.6	16.9
ATP, 8 mM	0.1	2.9
4 mM	<0.1	4.7
0.8 mM	<0.1	7.3

TABLE IV

EFFECT OF THE TEMPERATURE ON THE ACTIVATION OF PURIFIED PHOSPHORYLASE PHOSPHATASE

The purified phosphorylase phosphatase obtained after perfusion with NaF or NaCl was incubated for 2 min at 15° and 25° in the presence of 0.2 mM ATP, 0.25 mM $MgSO_4$ and 12 mM Tris (pH 7.4). The reaction was stopped by diluting the incubation mixture with cold 10 mM Tris buffer (pH 7.4), followed immediately by the assay of phosphorylase phosphatase. The data with the calculated standard error of the mean (n = 16) are expressed as a percentage of the full activation.

Perfusion	*% Activation*		Q_{10}
	15°	*25°*	
NaF	24.3 ± 1.3	33.6 ± 2.2	1.38
NaCl	28.1 ± 3.0	39.5 ± 4.6	1.41

Table IV shows that the activation of both enzyme preparations possessed the same temperature dependency since the percentage activation and the calculated Q_{10} were very similar.

The effect of pH on the activation of the phosphorylase phosphatase preparations, purified after perfusion with NaCl and NaF are represented in Fig. 3. The pH optimum for activation was found to be about 7.5 in both preparations.

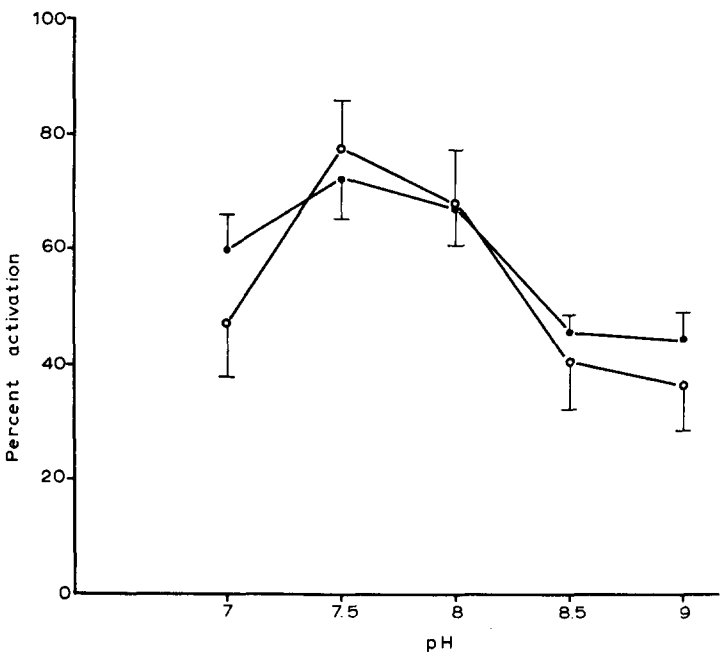

Fig. 3. Effect of pH on the activation of purified phosphorylase phosphatase. Both enzyme fractions obtained after perfusion of the dog liver with NaF (●—●) or NaCl (○—○) were incubated for 2 min at 25° in the presence of 0.1 mM ATP, 0.12 mM $MgSO_4$ and 20 mM Tris at the indicated pH values. The reaction was stopped by diluting the incubation mixture with cold 40 mM Tris. Thus all incubation mixtures were brought to pH 7.4 before assaying. The data are expressed as percentages of the full activation observed at pH 8. Vertical bars represent the S.E. (n = 14 to 20).

DISCUSSION

In the present study we have shown that perfusion of dog liver with 0.2 M NaF produces a reversible inactivation of phosphorylase phosphatase. Inactivation by NaF and reactivation by ATP in the presence of Mg^{2+}, could also be observed in dialyzed extracts; these properties were time, temperature and concentration dependent. All these interconversions were of a stable character because the resulting activity was maintained even after a several hundredfold dilution, precipitation of the enzyme with $(NH_4)_2SO_4$ and dialysis.

It seems appropriate to recall here that NaF has previously been described by WOSILAIT AND SUTHERLAND[5] as an inhibitor of the phosphorylase phosphatase reaction. This inhibitory effect appears unrelated to the inactivation since it can be observed under conditions where no inactivation occurs.

In some instances only a slight activation resulted on addition of ATP and Mg^{2+} in dog liver homogenates obtained after perfusion with NaCl. Since very low concentrations of ATP and $MgSO_4$ are sufficient to obtain activation, this effect could occur during preparation. Preliminary experiments (Kalala, Goris and Merlevede, unpublished observations) did not show any influence of hormones (insulin, adrenalin, glucagon) on mouse liver phosphorylase phosphatase. This could also be due to a rapid *post mortem* activation, or might indicate that activation in the presence of ATP and Mg^{2+}, which resembles the effect obtained in adrenal cortex[6] and in muscle[7], is not affected by hormones. As shown for glucocorticoids in mouse liver[8], the important change in phosphorylase phosphatase activity was very probably not due to this mechanism.

All of the investigated properties of the inactive enzyme, purified after perfusion of the liver with NaF or NaCl, appear to be identical.

(a) Similar concentrations of ATP and Mg^{2+} were necessary to obtain activation of the phosphatase, and in both cases 5 mM Mg^{2+} induced only a small activation. The time course of activation was similar for both enzyme fractions. In both preparations inactivation was also observed with ATP alone.

(b) The percent activation observed at 15° and 25°, and the calculated Q_{10} values were very similar in both cases. The order of magnitude observed for the Q_{10} is compatible with an enzymatic mechanism[9].

(c) The pH optimum was found to be about 7.5 for the activation of both enzyme fractions.

NaF has been described as stimulating adenylcyclase activity[10], and cyclic AMP decreases phosphorylase phosphatase activity in preparations obtained from adrenal cortex[11] and muscle[7]. Since no effect of the cyclic nucleotide could be shown in homogenates obtained from livers after NaCl perfusion, an inactivation by this combined mechanism seems improbable. The possibility that a spontaneous activation of liver phosphorylase phosphatase by ATP and Mg^{2+} occurs during preparation of the enzyme fraction, and that this activation could be prevented by NaF, can hardly be considered, since activation with ATP and Mg^{2+} could still be obtained in the presence of NaF. Moreover, inactivation with NaF was observed in the absence of ATP and Mg^{2+} which are necessary for adenyl cyclase activity or activation of the phosphatase. However, the possibility that the NaF inactivation of phosphorylase phosphatase is caused by interference with Mg^{2+} on the enzyme cannot be excluded.

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