BBA 75545

STUDIES ON THE PARTIAL REACTIONS CATALYZED BY THE $(Na^+ + K^+)$-ACTIVATED ATPase

II. EFFECTS OF OLIGOMYCIN AND OTHER INHIBITORS OF THE ATPase ON THE *p*-NITROPHENYLPHOSPHATASE

A. ASKARI AND D. KOYAL

Department of Pharmacology, Cornell University Medical College, New York (U.S.A.)

(Received June 22nd, 1970)
(Revised manuscript received September 21st, 1970)

SUMMARY

1. It is known that the K^+-activated *p*-nitrophenylphosphatase, in contrast to $(Na^+ + K^+)$-activated ATPase, is not inhibited by oligomycin; and that when *p*-nitrophenylphosphatase is activated by $Na^+ + K^+ + ATP$, the activity becomes partially sensitive to oligomycin. The object of this work was the further study of the mechanism of action of oligomycin on the enzyme complex.

2. It was shown that oligomycin blocks the stimulation of the activity that is caused by $Na^+ + ATP$. The K^+-dependent portion of the activity remained insensitive to oligomycin.

3. In the presence of Na^+ and low concentrations of K^+, oligomycin stimulated the *p*-nitrophenylphosphatase. This effect of oligomycin, like the activating effect of ATP, was accompanied by a decrease in the apparent K_m of K^+.

4. The curve of activation of enzyme as a function of oligomycin concentration had a sharp peak. Maximum activation obtained with oligomycin never approached that obtained with ATP. It was concluded that Na^+-dependent binding of either ATP or oligomycin to a modifying site affects the interaction of K^+ with the enzyme, and that higher concentrations of oligomycin block the modifying effects of ATP and lower oligomycin concentrations.

5. Ethacrynic acid, chlorpromazine, *N*-ethylmaleimide, and Dio-9 inhibited the $(Na^+ + K^+)$-activated ATPase and the K^+-activated *p*-nitrophenylphosphatase. In this respect these inhibitors are more similar to ouabain than to oligomycin.

INTRODUCTION

Several studies with the partially purified preparations of the $(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) have indicated that a K^+-stimulated *p*-nitrophenylphosphatase activity is intimately related to the ATPase activity and may represent the terminal step of the sequence of the reactions leading to ATP hydrolysis[1]. The exact relation of *p*-nitrophenylphosphatase activity to

ATPase activity is not known. The two activities may be catalyzed by the same enzyme protein or, as suggested by studies on the effect of *p*-nitrophenylphosphate on Na^+ transport in red cell ghosts, *p*-nitrophenylphosphatase may be one of the two distinct enzymes that are involved in the over-all ATPase activity[2]. The finding of ISRAEL AND TITUS[3] that the K^+-activated phosphatase, in contrast to the $(Na^+ + K^+)$-activated ATPase, is not inhibited by oligomycin, suggested that the determination of the site of action of this inhibitor within the enzyme complex may be of help in clarifying the relation of the various activities to each other. Subsequent studies of our laboratory which showed that in the presence of Na^+ and ATP the K^+-activated *p*-nitrophenylphosphatase becomes partially sensitive to oligomycin led us to propose a scheme for the partial reactions catalyzed by this enzyme complex, and suggest a site for the action of oligomycin[4]. The object of the present work was the further study of the mechanism of action of oligomycin on the enzyme complex, and the comparison of the action of oligomycin with those of some other inhibitors of the $(Na^+ + K^+)$-activated ATPase. A preliminary account of portions of this work has been published[5].

MATERIALS AND METHODS

The enzyme was prepared and assayed as described before[4,6]. Substrates, oligomycin, and ouabain were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ethacrynic acid was obtained from Merck Institute for Therapeutic Research (West Point, Pa.). Dio-9 was generously provided by Dr. R. J. Guillory, Section of Biochemistry and Molecular Biology, Cornell University.

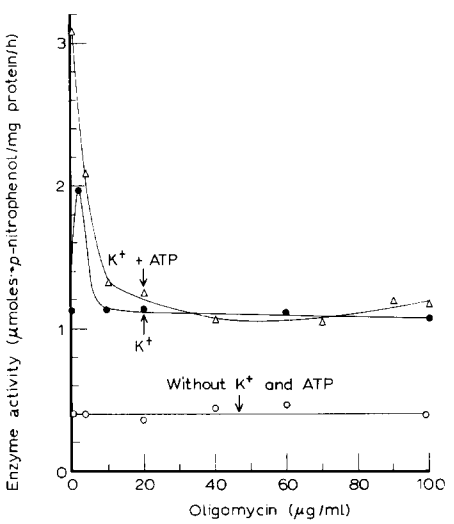

Fig. 1. Effects of varying concentrations of oligomycin on *p*-nitrophenylphosphatase activity. All reaction mixtures contained 4 mM substrate, 4 mM Mg^{2+} and 20 mM Na^+. ○, without K^+ and ATP; ●, 0.8 mM K^+; △, 0.8 mM K^+ + 0.1 mM ATP.

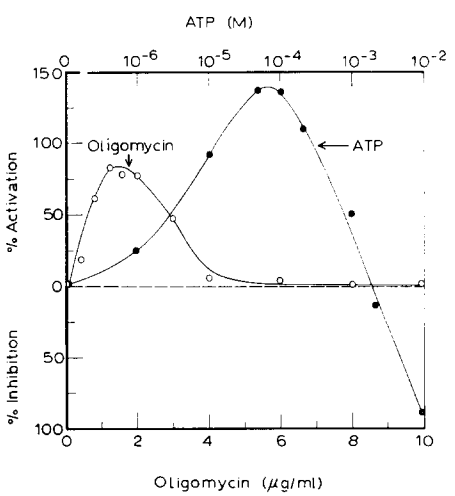

Fig. 2. Comparison between the activating effects of oligomycin (○) and ATP (●) on *p*-nitrophenylphosphatase activity in the presence of 20 mM Na^+ and 0.5 mM K^+. All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}.

RESULTS

Inhibition of p-nitrophenylphosphatase by oligomycin

Our previous data[4] and those of INTURRISI AND TITUS[7] clearly showed that while oligomycin inhibits the ($Na^+ + K^+$)-activated ATPase, it does not affect the K^+-activated *p*-nitrophenylphosphatase. We demonstrated, however, that when the K^+-activated *p*-nitrophenylphosphatase was further stimulated by the presence of Na^+ and ATP the enzyme activity became partially sensitive to oligomycin[4]. These results can be interpreted in two different ways: (1) Oligomycin interferes with the activating effect of Na^+ + ATP on the enzyme. (2) The enzyme acquires oligomycin sensitivity only in the presence of Na^+ and ATP. Although we have already expressed preference for the first possibility[4], our previous data were not extensive enough to rule out the second. The experiments of Fig. 1 were designed to test the above alternatives. The data show that in the presence of Na^+, K^+ and ATP while increasing concentrations of oligomycin completely block the activating effects of Na^+ and ATP, the enzyme activity never drops to the level that is obtained in the absence of K^+. Clearly the K^+-activated *p*-nitrophenylphosphatase does not become sensitive to oligomycin even in the presence of Na^+ and ATP.

Activating effect of oligomycin on p-nitrophenylphosphatase

As the data of Fig. 1 show, in the presence of Na^+ and K^+ lower concentrations of oligomycin have an activating effect on enzyme activity. More extensive data on the relation of oligomycin concentration to this activating effect, and comparison between the activating effects of oligomycin and ATP on the activity are shown in Fig. 2. It has been shown[6,8] that the activating effect of ATP is demonstrable only in the presence of suboptimal concentrations of K^+, and that this is due to a decrease in the apparent K_m of K^+ in the presence of Na^+ and ATP. The following experiments were performed to determine if the mechanism of activating effect of oligomycin were similar to that of ATP. Fig. 3 shows the effects of varying concentrations of K^+ on the enzyme activity in the presence and absence of Na^+ and oligomycin. From the data it is apparent that (a) in agreement with the previous results[6] Na^+ decreases the v_{max} and increases the apparent K_m for K^+; (b) in the absence of Na^+, oligomycin does not affect the [K^+]–velocity curve; and (c) in the presence of Na^+ addition of oligomycin does not alter the v_{max}, but it lowers the apparent K_m of K^+. It may appear from the above data that the apparent activating effect of oligomycin is simply due to its ability to overcome the effect of Na^+ on the apparent K_m of K^+. Data of Fig. 4 which shows a more detailed analysis of a small portion of Fig. 3, show that this is not the case. The apparent K_m of K^+ in the presence of oligomycin and Na^+ is not only lower than the K_m in the presence of Na^+, but also significantly lower than the K_m in the absence of Na^+. Fig. 5, shows the effect of varying concentrations of Na^+ on the activating effect of oligomycin in the presence of a fixed concentration of K^+. It is obvious that there is an optimum concentration of Na^+ for the activating effect of oligomycin.

The combined data of Figs. 3, 4, and 5 clearly show that the activating effect of low concentrations of oligomycin on the enzyme is quite similar to the effects of ATP and other nucleoside triphosphates on the enzyme. As evident from Fig. 2 the two major differences between oligomycin and ATP are that (a) the maximum acti-

vation obtained with oligomycin never approaches that obtained with ATP; and (b) higher concentrations of ATP, unlike oligomycin, can inhibit the K^+-activated *p*-nitrophenylphosphatase.

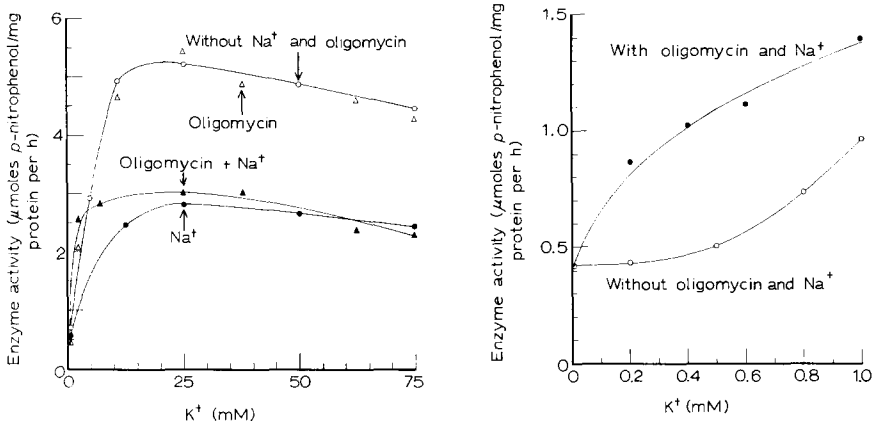

Fig. 3. Effects of varying concentrations of K^+ on *p*-nitrophenylphosphatase in the presence and absence of 20 mM Na^+ and oligomycin (2 μg/ml). All reaction mixtures contained 4 mM substrate and 4 mM Mg^{2+}. ○, without Na^+ and oligomycin; ●, Na^+; △, oligomycin; ▲, Na^+ and oligomycin.

Fig. 4. Effects of varying concentration of K^+ on the *p*-nitrophenylphosphatase in the presence (●) and absence (○) of Na^+ and oligomycin. Reaction conditions were the same as described for Fig. 3.

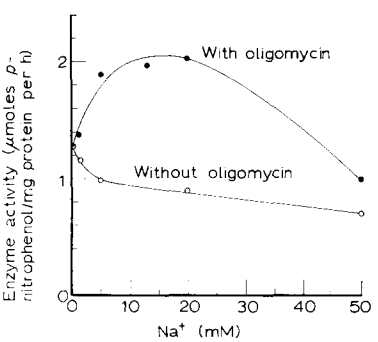

Fig. 5. Effects of varying concentration of Na^+ on the *p*-nitrophenylphosphatase activity in the presence (●) and absence (○) of oligomycin (2 μg/ml). All reaction mixtures contained 4 mM substrate, 4 mM Mg^{2+} and 0.7 mM K^+.

Comparison between the effects of oligomycin and ouabain

It is known that cardiac glycosides, in contrast to oligomycin, inhibit the K^+-activated *p*-nitrophenylphosphatase[1]. This fact indicates that cardiac glycosides have certain actions on the enzyme complex that are not shared by oligomycin. It was of interest to find out, however, if an activating effect on the enzyme similar to that of oligomycin could be obtained with cardiac glycosides. Extensive experiments similar to those of Figs. 1–5 were performed with ouabain. Under a variety of tested conditions no activating effect of ouabain could be demonstrated.

TABLE I

EFFECTS OF SEVERAL INHIBITORS OF $(Na^+ + K^+)$-ACTIVATED ATPase ON THE *p*-NITROPHENYLPHOSPHATASE

The various activities and the effects of inhibitors were determined as described before[4].

	Concentration necessary for half-maximal inhibition of:		
	$(Na^+ + K^+)$-activated ATPase	*K^+-activated p-nitrophenylphosphatase*	*$(Na^+ + K^+ + ATP)$-activated p-nitrophenylphosphatase*
Ethacrynic acid	$1 \cdot 10^{-4}$ M	$5 \cdot 10^{-4}$ M	$2 \cdot 10^{-4}$ M
Chlorpromazine	$5 \cdot 10^{-4}$ M	$5 \cdot 10^{-4}$ M	$5 \cdot 10^{-4}$ M
N-ethylmaleimide	$3 \cdot 10^{-4}$ M	$1 \cdot 10^{-3}$ M	$3 \cdot 10^{-4}$ M
Dio-9	20 μg/ml	20 μg/ml	20 μg/ml

Effects of other inhibitors of $(Na^+ + K^+)$-activated ATPase on p-nitrophenylphosphatase

In experiments similar to those of Fig. 1 the effects of the compounds listed in Table I on *p*-nitrophenylphosphatase were studied. None of the tested compounds had an activating effect similar to that of oligomycin. With all the tested compounds complete inhibition of $(Na^+ + K^+)$-activated ATPase, the K^+-activated *p*-nitrophenylphosphatase and the $(Na^+ + K^+ + ATP)$-activated *p*-nitrophenylphosphatase could be obtained. Table I shows the concentration of each compound necessary for half-maximal inhibition of the above activities. The effects of rutamycin on *p*-nitrophenylphosphatase were also studied and found to be similar to those of oligomycin.

DISCUSSION

On the basis of the results of our previous studies[4] we proposed the existence of a modifying site on the $(Na^+ + K^+)$-activated ATPase complex for the binding of a nucleoside triphosphate. A consequence of this binding, which requires Na^+, was shown to be a decrease in the apparent K_m of the activator cation K^+ (refs. 6 and 8). It was tentatively suggested[4] that oligomycin either interferes with the binding of ATP to the modifying site, or blocks the modifying process which leads to increased activating effect of K^+. The studies presented in this paper suggest that the two possibilities are not mutually exclusive. The most likely explanation of the activating effects of oligomycin is that the antibiotic binds to the same modifying site that is involved in the binding of nucleoside triphosphates, and that the Na^+-dependent binding of oligomycin also leads to an increase in the activating effects of low concentrations of K^+. The inhibitory effect of oligomycin on the ATP activation can be explained by the shape of the curve of activation of the enzyme as a function of oligomycin concentration. The sharp peak of this curve shows that increasing concentrations of oligomycin interfere with the modifying effects of the lower concentrations of oligomycin and ATP.

Although the above hypothesis is consistent with the data, it is appropriate to consider other possible explanations for the effects of oligomycin on *p*-nitrophenylphosphatase activity. As we have pointed out before[4,6] it may be assumed that $(Na^+ + ATP)$-activation of the enzyme is not due to the binding of ATP to a modi-

fying site, but rather due to the Na^+-dependent formation of the phosphorylated enzyme which modifies the enzyme in a way that is more favourable to the phosphatase activity. In spite of our several previous objections to this assumption[4] (*e.g.* the ability of CTP to produce activation), it is important to see if the data on oligomycin can be correlated with the assignment of a modifying role to the phosphorylation process. In experiments where the activation of *p*-nitrophenylphosphatase with oligomycin is obtained, the only possible sources for the production of phosphorylated enzyme are nitrophenylphosphate and the orthophosphate that is formed from the substrate. In the presence of cardiac glycosides the formation of phosphorylated enzyme from orthophosphate[9–11] and nitrophenylphosphate[12] has been demonstrated. It is not known whether oligomycin can induce the incorporation of orthophosphate into the enzyme, but the limited available evidence[12] indicates that oligomycin, unlike ouabain, does not cause the formation of the phosphorylated enzyme from *p*-nitrophenylphosphate. In view of this and the previous arguments[4], with the present evidence we favour the hypothesis of the existence of a common modifying site for ATP and oligomycin.

The inability of ouabain to activate *p*-nitrophenylphosphatase in the presence of Na^+ and low concentrations of K^+, and its ability to inhibit K^+-activated *p*-nitrophenylphosphatase strongly suggest that ouabain does not react with the hypothetical ATP–oligomycin site. Our studies on the other inhibitors of $(Na^+ + K^+)$-activated ATPase reveal that only rutamycin has effects on the enzyme complex similar to those of oligomycin (*cf.* INTURRISI AND TITUS[7]). Inhibition of $(Na^+ + K^+)$-activated ATPase by ethacrynic acid[13], chlorpromazine[14,15] and *N*-ethylmaleimide[16] has been reported. The antibiotic Dio-9 was tested because of its inhibitory and uncoupling effects on oxidative phosphorylation[17]. All of these compounds inhibited K^+-activated *p*-nitrophenylphosphatase and $(Na^+ + K^+)$-activated ATPase. In this respect they are more similar to cardiac glycosides than to oligomycin.

ACKNOWLEDGEMENT

This investigation was supported by U.S. Public Health Service Grant HE-10884 from the National Heart Institute.

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