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On the Inhibition of (Na^+ + K^+)-Activated Adenosine Triphosphatase by Diisopropyl Fluorophosphate

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SUMMARY

The (Na^+ + K^+)- activated transport ATPase from guinea pig kidney cortex is inhibited in the presence of high concentrations of diisopropyl fluorophosphate (DFP) if Mg^{++} is present, although the inhibition is caused not by DFP but by the fluoride released in its hydrolysis. Fluoride at 1 mM inhibits transport ATPase in a few minutes. The second-order rate constant for inhibition is 2.7×10^2 M^{-1} min^{-1}. Mg^{++} is necessary for the inhibition and K^+ markedly increases the rate of inhibition, but Na^+ is without effect. ATP prevents inhibition by fluoride, and fluoride inhibition can be reversed slowly. ^{14}C-DFP even without Mg^{++} rapidly labels one or more proteins in the ATPase preparation, but there is no evidence that these proteins are involved in the ATPase activity.

INTRODUCTION

Part of the ATPase activity found in the membrane and microsomal fractions of cell homogenates is dependent upon the presence of sodium and potassium ions and is inhibited by ouabain. Because this activity is believed to be associated with the transport of these ions, the enzyme is referred to as sodium- and potassium-activated transport ATPase (EC 3.6.1.4) (1, 2). It is thought that a phosphoryl-enzyme intermediate is formed during the hydrolysis of ATP, and several groups of workers have obtained a labeled phosphoprotein by incubating the enzyme preparation with labeled [γ-^{32}P]ATP (3, 4). On the basis of kinetic observations, Fahn *et al.* (5) proposed that there are two phosphoryl-enzyme intermediates, one having a high-energy bond and the other a low-energy bond. Hokin and Yoda (6) obtained ^{32}P-labeled *O*-phosphorylserine by hydrolysis of an enzyme preparation that had been treated with ^{32}P-labeled diisopropyl fluorophosphate. They reported that DFP[2] irreversibly inhibits transport ATPase and that this inhibition is prevented by ATP. Thus these authors concluded that this particular serine residue is involved in the hydrolysis of ATP. The amino acid residue that is phosphorylated with ^{32}P-ATP is not serine but is thought to be glutamic acid (7). Thus two different phosphoproteins can be obtained and have been separated (8). The protein derived by reaction with DFP is of course a diisopropylphosphoryl-protein and, by reaction with ATP, a phosphoryl-protein. The two proteins have been considered components of transport ATPase (8).

Inhibition of ATPase by DFP has also been reported by Sachs *et al.* (9). In our

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[2] The abbreviation used is: DFP, diisopropyl fluorophosphate.

investigation of the inhibition of transport ATPase by DFP we found that there was a considerable lag period before inhibition developed. This led us to suspect that DFP itself might not inhibit ATPase but that fluoride, both as an impurity present in the DFP and derived continuously from its hydrolysis, might be responsible for the inhibition. This turned out to be the case. Inhibition of ATPase by fluoride has been reported by Opit *et al.* (10), Yoshida *et al.* (11), and Sachs *et al.* (9).

MATERIALS AND METHODS

Guinea pig kidney cortex microsomal ATPase (12) was prepared with the following modifications. Sliced kidney cortices (20 g) were extruded with a French press at 6000 psi, and the resulting paste was homogenized with a Teflon pestle in a Potter-Elvehjem homogenizer to a final volume of 320 ml in 0.25 M sucrose containing 20 mM NaCl, 5 mM disodium EDTA, 1 mM $MgCl_2$, and 10 mM imidazole, pH 7.0. The homogenate was centrifuged at 700 × *g* for 10 min, and the supernatant fluid was centrifuged for 1 hr at 39,000 × *g*. The resulting sediment was rehomogenized in a Potter-Elvehjem homogenizer to a final volume of 320 ml in 0.25 M sucrose containing 2 mM disodium EDTA, 0.1 mM $MgCl_2$, and 4 mM imidazole, pH 7.0. The homogenate was then centrifuged at 12,000 × *g* for 30 min. The resulting supernatant fraction was centrifuged at 120,000 × *g* for 30 min. The sediment was suspended in 10 mM imidazole HCl buffer, pH 7.0, containing 0.1 mM disodium EDTA. The suspended material was washed three times in the same buffer by centrifugation at 120,000 × *g* and finally stored at 4°. It was stable for an indefinite period at that temperature. The protein yield was about 120 mg, with a specific activity of about 50 μmoles of P_i per milligram per hour; 90% of the ATPase activity was inhibited by ouabain. Suspension of the 120,000 × *g* sediment in 1 M urea did not improve the ratio of ouabain-sensitive to ouabain-insensitive ATPase.

Preliminary incubations with fluoride or DFP were carried out at 37°. The incubation vessel contained 10 mM imidazole HCl buffer, pH 7.0, and approximately 4 mg of protein in a total volume of 1.0 ml, including all other additions as indicated. An aliquot (50 μl) of the incubation mixture was added direct to 0.95 ml of ATPase reaction mixture at 37° at the indicated times. The ATPase assays were carried out in reaction mixtures consisting of 3 mM disodium ATP, 5 mM $MgCl_2$, 0.1 M NaCl, 20 mM KCl, 0.5 mM disodium EDTA, 20 mM imidazole, and 20 mM glycylglycine, with or without 0.1 mM ouabain. The reaction was stopped at 4.5 min by adding 0.5 ml of 1.2 M perchloric acid containing 80 mg of silicotungstic acid per milliliter. Protein was measured by the method of Lowry *et al.* (13).

^{14}C-DFP (111 mCi/mmole) was obtained from New England Nuclear Corporation. Unlabeled DFP was a product of Aldrich Chemical Company. Imidazole was recrystallized from benzene, and all other chemicals were standard reagent grade.

RESULTS

Our preparation of microsomal ATPase from guinea pig kidney cortex is readily inhibited by 1 mM fluoride, provided that Mg^{++} is present (Fig. 1). In agreement with Opit *et al.* (10), we found that in the absence of Mg^{++} no inhibition is observed; the addition of K^+ increases the rate of inhibition quite markedly, but Na^+ does not affect the rate. The initial rate of inhibition is quite rapid, but then slows more quickly than anticipated for a pseudo-first-order reaction and does not readily approach completion; i.e., it does not readily approach the limit of ouabain inhibition. First-order kinetics of inhibition is obtained, however, if we assume that 80% of the transport ATPase is subject to rapid inhibition, and that the remaining 20% is a different transport ATPase, not readily inhibited by fluoride (Fig. 2). (We use the term transport ATPase in the operational sense of ATPase activity which is dependent upon Na^+ and K^+ and is inhibited by ouabain.) The pseudo-first-order rate constants are proportional to the concentration of fluoride in the range 0.5-2.0 mM. Thus we conclude that the inhibition reaction is second-order and the rate constant has the

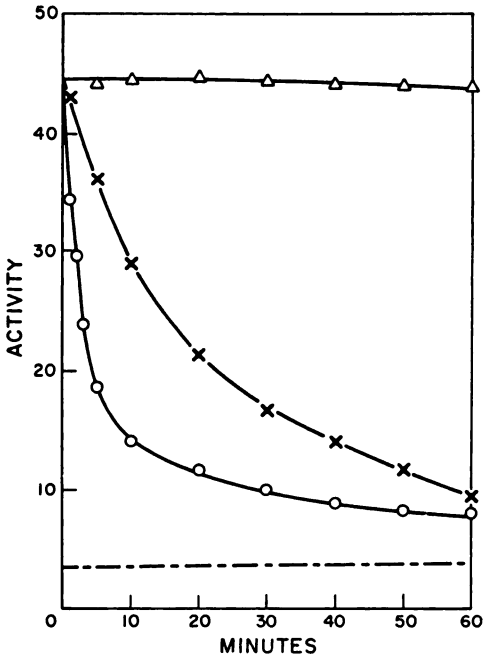

FIG. 1. *Effect of Mg^{++} and K^+ on inhibition by fluoride of ATPase of guinea pig renal cortex membranes*

Inhibition was studied at 37° in 10 mM imidazole buffer, pH 7.0. Other additions were: △, 1 mM F^- and 10 mM KCl; ×, 1 mM F^- and 15 mM $MgCl_2$; ○, 1 mM F^-, 10 mM KCl, and 15 mM $MgCl_2$. After the indicated time intervals, aliquots of these incubation mixtures were diluted 20-fold and assayed for residual ATPase activity as described under MATERIALS AND METHODS. The assay times were 4.5 min in all cases. The rate of inhibition by fluoride in the presence of Mg^{++} was not increased by Na^+. The dashed line represents ATPase activity in the presence of 0.1 mM ouabain in the assay mixture containing untreated enzyme and also the enzyme incubated with F^-, Mg^{++}, and K^+ as described above. The ATPase activity is expressed in terms of μmoles of P_i per mg of protein per hr.

value 2.7×10^2 M^{-1} min^{-1} at pH 7.0 and 37° in the presence of Mg^{++} and K^+.

At low fluoride levels, 0.1 mM, the rate of inhibition is anomalous. There is a lag period of about 20 min, followed by pseudo-first-order inhibition with a calculated second-order rate constant less than half as great as that observed at higher fluoride concentrations (Fig. 3).

The effect of pH on fluoride inhibition was studied to a very limited extent. At pH 6 the enzyme itself was quite unstable, but fluoride inhibition could still be easily demonstrated. However, it is difficult to compare the rates of inhibition at pH 6 and pH 7. At pH 8.0 the rate of inhibition is about one-third less rapid than at pH 7.0.

No fluoride inhibition is obtained in the absence of Mg^{++}. The rate of inhibition by 1 mM fluoride is half-maximal at 0.3 mM Mg^{++} and depends upon the Mg^{++} concen-

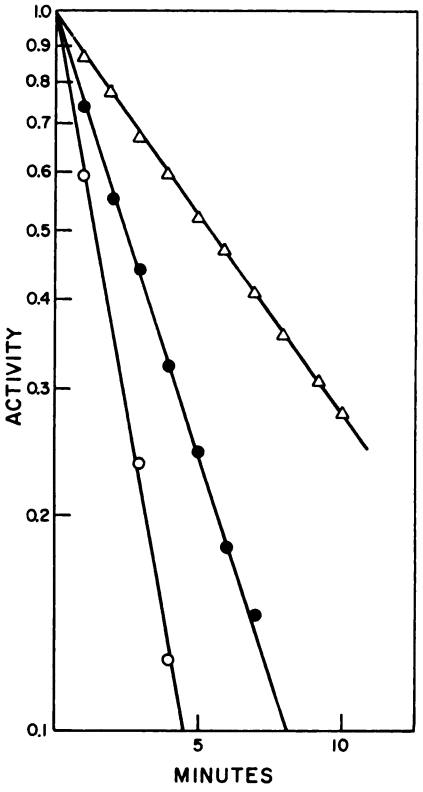

FIG. 2. *Semilogarithmic plot of time course of inhibition by fluoride of guinea pig renal cortex membrane ATPase*

The initial activity is designated as 1. The incubations were carried out at 37° in the presence of 10 mM KCl and 15 mM $MgCl_2$ in 10 mM imidazole buffer, pH 7.0. The fluoride concentrations were 2 mM (○), 1 mM (●), and 0.5 mM (△). The residual enzyme activity was measured at the indicated times as described under MATERIALS AND METHODS. The assay times were 4.5 min. In the calculations 80% of the total ATPase activity was assumed to be readily susceptible to inhibition by fluoride. The remaining 20% of the activity consists of one-half that is not inhibited by ouabain and one-half that is not readily inhibited by fluoride.

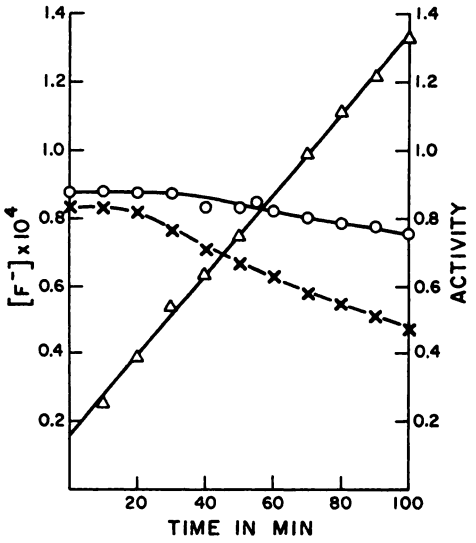

FIG. 3. *Time course of inhibition by fluoride or DFP of ATPase of guinea pig renal cortex membranes*

The graph shows ATPase activity at the indicated time intervals after incubation of the enzyme at 37° with 15 mM $MgCl_2$ and 10 mM KCl in 10 mM imidazole buffer, pH 7.0, with the addition of 0.1 mM F^- (×) or 3 mM DFP (○). The molar concentration of fluoride developing in the reaction system with DFP is also shown (△). The ATPase activity was measured with 0.2 mg of protein per ml of assay medium at the indicated incubation times. The activity is given in terms of change in optical density at 650 mμ with an assay time of 4.5 min. An optical density of 1.16 corresponds to 1 μmole of P_i per ml of assay medium.

tration in the usual hyperbolic manner, consistent with the binding of 1 molecule of Mg^{++} by ATPase with a dissociation constant of 0.3 mM. It is also possible that MgF^+ is the binding species and/or the active inhibitor. The dissociation constant of MgF^+ is about 0.05 M, and under our experimental conditions about 2% of the Mg^{++} exists as the fluoride complex. Fluoride inhibition is completely prevented by 1 mM ATP (in the presence of 15 mM Mg^{++} and 10 mM KCl). We did not investigate the kinetics of the inhibition by fluoride of the *p*-nitrophenyl phosphatase activity of our preparation. However, we did note that in the presence of Mg^{++} and K^+, 1 mM fluoride completely inhibited the *p*-nitrophenyl phosphatase activity of these preparations in 10 min.

When incubated with 3 mM DFP, the same concentration as used by others, ATPase is inhibited only slightly or not at all during the first 30 min, but a slow rate of inhibition becomes apparent after this time (Fig. 1). This observation is in accordance with expectations if a low concentration of fluoride were initially present (since low concentrations of fluoride show a lag period), or if fluoride were formed during the incubation. Only a small percentage of the DFP has to be hydrolyzed spontaneously in 1 hr (14). We set up our preparation with a fluoride electrode to measure fluoride ion concentration. We found an initial fluoride concentration of 20 μM, which increased with time and reached 100 μM after 70 min. After 30 min the onset of a slow inhibitory reaction is apparent. In the absence of Mg^{++} no inhibitory

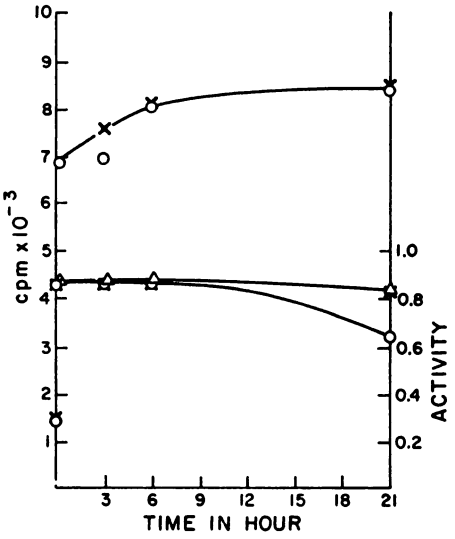

FIG. 4. *Labeling of guinea pig renal cortex membrane ATPase by ^{14}C-DFP*

The graph shows the amount of labeling of protein (counts per minute per milligram) and the activity of ATPase as a function of the time of prior incubation with 0.1 mM ^{14}C-DFP in 10 mM imidazole, pH 7.0, at 37°. The uppermost curve represents labeling of protein in the presence (○) and absence (×) of 15 mM $MgCl_2$ and 10 mM KCl. The lower curves represent enzyme activity with 0.1 mM ^{14}C-DFP in the presence (○) and absence (×) of 15 mM $MgCl_2$ and 10 mM KCl, and control activity in the presence of 15 mM $MgCl_2$ and 10 mM KCl but without ^{14}C-DFP (△). The ATPase activity is expressed in terms of the change in optical density as explained in the legend to Fig. 3.

reaction occurs. It is perfectly clear that the slow inhibition of ATPase that occurs in the presence of DFP can be readily explained as inhibition resulting from the presence of fluoride that is released by the slow hydrolysis of DFP. There is no evidence whatsoever that DFP per se, even at 3 mM concentration, produces any significant inhibition.

Hokin and Yoda (6) labeled beef kidney cortex microsomes with 3 mM DFP and Chignell and Titus (8) labeled rat kidney cortex microsomes. Any number of proteins might be labeled by incubation with high concentrations of DFP. The critical point is whether these proteins have anything to do with the ATPase system. Our finding that DFP does not inhibit ATPase leads us to conclude that any proteins labeled with DFP cannot be part of the ATPase system. Because of the importance of this question, we performed some labeling experiments to see whether any evidence would emerge suggesting that DFP might label a protein involved in ATPase. To start, we labeled our preparation with ^{14}C-DFP using 11 μM and 3.6 μM concentrations. In both cases labeling was rapid, being completed within 1–2 min, and we obtained about 8×10^3 cpm/mg of protein. The labeling with 3.6 μM DFP was the same in the presence of 15 mM Mg^{++} and 10 mM K^+ as in the absence of these ions, and was unaffected by the addition of 1 mM ATP in the presence of Mg^{++} and K^+.

The very rapid labeling obtained with 3.6 μM DFP suggested that an esterase was present in the preparation. This was checked using phenyl acetate as a substrate, and an esterase activity of 2.5 μmoles/mg of protein per minute was found. This esterase activity was inhibited 85% in 10 min by 1 μM DFP, but no further inhibition was obtained. The extent of inhibition was not affected by Mg^{++}, K^+, Ca^{++}, or ATP (Table 1).

We wished to label at higher DFP concentrations, but 0.1 mM was the highest concentration feasible. Hokin and Yoda (6) used 3 mM ^{32}P-DFP, but partially offsetting their higher concentration was the fact that our DFP had a specific activity more than 3 times greater than theirs. They obtained 11,000 cpm/mg of protein. Since our specific activity of DFP was more than 3 times as high and, in addition, our enzymatic specific activity was 3 times as high, we should have been able to obtain 100,000 cpm/mg of protein. Thus, although we used a lower concentration of DFP, we should have obtained about the same number of counts that they did in about twice the time. The technique we used was first to add nonradioactive DFP, 10 μM, for 4 min to inhibit the esterase referred to above, followed by 0.1 mM radioactive DFP. About 6×10^3 cpm/mg were obtained within our first measurement at 3 min, and this value rose slowly to 9.5×10^3 cpm/mg in 21 hr. Although our initial label was sizable, it did not rise to anywhere near the value expected from the work of others if the material labeled were an essential component of ATPase. Equally important, the same labeling was obtained in the absence of Mg^{++} and K^+. Thus, under conditions that could have resulted in labeling of ATPase, we obtained no labeling attributable to ATPase. The labeling we did observe occurred under conditions in which there was no inhibition of ATPase, whether from F^- or DFP.

TABLE 1

Inhibition by DFP of esterase for phenyl acetate present in membranes of guinea pig renal cortex

This table shows the lack of effect of different concentrations of Mg^{++}, K^+, ATP, and Ca^{++} upon the labeling of protein by ^{14}C-DFP and the inhibition of esterase activity.

Additions to ^{14}C-DFP (3 μM)	Zero time		10 min	
	Radioactivity	Esterase	Radioactivity	Esterase
	cpm/mg	*μmoles/mg/min*	*cpm/mg*	*μmole/mg/min*
Mg^{++}, 15 mM; K^+, 10 mM	238	2.5	8992	0.33
No Mg^{++} or K^+	224	2.5	8402	0.28
Mg^{++}, 15 mM; K^+, 10 mM; ATP, 1 mM	190	2.5	9306	0.32
Mg^{++}, 3 mM; Ca^{++}, 10 mM	248	2.5	9119	0.30

DISCUSSION

DFP is a poor chemical phosphorylating agent, as evidenced by the fact that it hydrolyzes quite slowly. Nonetheless, DFP inhibits certain esterases and proteinases very readily. For example, an esterase in our

ATPase preparation was inhibited "immediately" in the presence of 3.6 μM DFP. The reason why DFP is so potent in inhibiting these esterases is that the reaction is catalyzed by the enzyme itself and the active site is phosphorylated in analogy with the normal acylation of the active site that occurs during the hydrolysis of esters.

The reported slow inhibition of $(Na^+ + K^+)$-transport ATPase at 3.6 mM DFP (6), in the absence of evidence to the contrary, must be taken as indicating an entirely different kind of reaction. Presumably some side chain functional groups are phosphorylated and the activity of the enzyme is adversely affected. The inhibition is obviously not the specific kind of active site reaction that occurs with esterases. We too find that ATPase is inhibited when incubated with DFP, but at a much slower rate than when it is inhibited by incubation with fluoride. In both cases, Mg^{++} is required. Since DFP preparations contain some fluoride and fluoride is formed continuously by the hydrolysis of DFP, it is necessary to rule out fluoride inhibition if one is to claim that DFP inhibits ATPase. Our measurements of ATPase inhibition in the presence of DFP, coupled with our simultaneous measurements of fluoride concentration, indicate that all the inhibition can be accounted for by the presence of fluoride produced from DFP by hydrolysis. Thus very little or no inhibition of ATPase arises from reaction with DFP per se.

The important question is whether some of the labeled protein obtained by reaction with DFP is part of the ATPase system or if it is all extraneous protein. If it were true that DFP inhibits ATPase, we would know that some of the labeled protein is associated with ATPase. When ATPase is incubated with DFP in the absence of Mg^{++} there is no inhibition, yet the labeling of protein is the same. Thus the phosphorylation of protein is independent of the inhibition of ATPase. This being the case, it would be illogical to assume that the labeled protein was a component of ATPase. These inhibition studies, which indicate that fluoride rather than DFP is the inhibitor, as well as the labeling studies, therefore provide no basis for the supposition that the labeled protein is related to ATPase activity.

These studies do not rule out the possibility that DFP may react silently with ATPase, i.e., to phosphorylate some group, without this change affecting the enzyme activity. If such a reaction were to occur, ATPase would be labeled. It would appear unlikely that much of the labeled protein arises in this way, because only a very small fraction of the protein is ATPase protein in these impure preparations. The important point of this discussion is that, based upon the present work, there is no evidence that DFP either inhibits or labels ATPase.

The mechanism of the progressive inhibition of $(Na^+ + K^+)$-transport ATPase by fluoride is completely unknown. The need for Mg^{++} can be interpreted in terms of the binding of this ion by some component of ATPase, and poses the question whether Mg^{++} might not be involved in some role besides complexation with ATP in the hydrolysis of ATP. On the other hand, it is possible that the need for Mg^{++} arises because it is not fluoride that is the inhibitor but MgF^+.

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