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Phosphatase and Ouabain-Sensitive Adenosine Triphosphatase Activities of the Perfused Frog Heart

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SUMMARY

An ATPase and *p*-nitrophenyl phosphatase activity has been characterized in the perfused frog ventricle. Addition of ATP and *p*-nitrophenyl phosphate to the perfusate results in their rapid hydrolysis upon contact with the ventricle. A spectrophotometric system was developed to monitor the perfusate continuously to determine the hydrolytic activity of the ventricle while also monitoring contraction. Ouabain at 3 μM significantly inhibits the ATPase during the positive inotropic action of the drug. This inhibition progressively increases as inotropism proceeds toward toxicity. Ten-fold higher concentrations of ouabain (30 μM) do not inhibit the *p*-nitrophenyl phosphatase. ATP increases the toxicity of digoxin and its binding to the ventricles. It is proposed that the ATPase activity of the ventricle might be associated with the ($Na^+ + K^+$)-dependent ATPase enzyme system

INTRODUCTION

Skou (1) has reviewed studies which indicate that the membrane-localized sodium-plus potassium-stimulated ATPase is the enzyme system responsible for the active transport of sodium and potassium ions in the cell. This enzyme is highly sensitive to inhibition by the digitalis glycosides, and it has been suggested that it might be the receptor responsible for the cardiotonic effect of these drugs (2). Although this enzymatic activity has been widely studied in fractionated heart muscle, studies in whole heart tissue have not, to our knowledge, been attempted. Perfused heart preparations are known to hydrolyze nucleotides and other phosphate-containing organic compounds introduced into the perfusate (3, 4), but the specificity and cellular origin of this activity are uncertain. The present investigation was undertaken to characterize this phosphatase activity further in intact, fully functional heart muscle. Intact frog heart ventricles were chosen because the gross and cellular simplicity of this preparation eliminates some of the ambiguities inherent in other cardiac preparations.

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METHODS

Frog ventricles from *Rana pipiens* hearts were mounted on the tips of Straub cannulae by thread tied around the atrioventricular groove and perfused in 2 ml of Ringer's solution containing 91.3 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.0 mM

$CaCl_2$, and 5.0 mM dextrose. The solution was gassed with 95% O_2–5% CO_2, pH 7.4, and the ventricles stimulated at 30/min with a 4-V square wave pulse of 1-msec duration. Perfusion consisted of back-and-forth movement of the Ringer's fluid between the ventricular cavity and cannula during contraction. The ventricles were allowed to equilibrate for 1 hr before experiments were started. Experiments were done at room temperature, which was maintained between 23° and 25°. In some experiments a new technique was developed wherein continuous monitoring of the perfusion fluid absorbance in a quartz 1-cm-square cannula was made possible by mounting the cannula in the light path of a Cary 15 recording spectrophotometer (Fig. 1). The solution was gassed through a No. 30 hypodermic needle. Neither gassing nor movement of fluid in the cannula interfered with the optical recording. In order to record contractions (stroke volume) simultaneously, the tip of the cannula holding the ventricle was inserted in an airtight chamber connected to a plethysmographic transducer and electronic pen writer.

All reagents used were of the highest grade available. Phosphate compounds were introduced as solutions neutralized to pH 7.4 with NaOH. Crystalline pyruvic kinase and lactic dehydrogenase enzyme suspensions in 2.2 M ammonium sulfate were obtained from Sigma Chemical Company and Calbiochem, respectively. Before use, the enzymes were centrifuged, the ammonium sulfate was removed, and the enzyme was taken up in an equal volume of 25 mM sodium phosphate buffer, pH 7.4. Inorganic phosphate was determined by the method of Fiske and SubbaRow (5) in Ringer's fluid at timed intervals after the addition of phosphate-containing compounds. Magnesium chloride (3.5 mM) was added to the Ringer's solution when pyruvic kinase was used and in the ^{3}H-digoxin and [α-^{32}P]ATP labeling experiments. Although ATP and other phosphate substrates introduced into the perfusion solution have the potential to change the pH of the solution, continuous monitoring of the pH with a glass pH electrode during the course of experiments showed no change in pH from 7.4. ^{3}H-Digoxin was kindly supplied by Burroughs Wellcome and Company, Tuckahoe, N. Y. (100 Ci/mole).

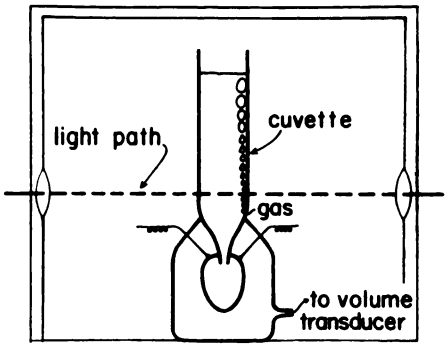

FIG. 1. *Diagrammatic representation of a frog ventricle mounted in the spectrophotometer*

The cannula was mounted in the light path to monitor the absorbance of the perfusate. The ventricle was electrically stimulated by the platinum point electrodes, and the stroke volume was monitored by changes in the chamber surrounding the ventricle.

In the labeling experiments, ventricles were perfused with 3 μM ^{3}H-digoxin in Ringer's solution alone or with ATP, AMP, or epinephrine for 3 min as indicated. The hearts were then given three 30-sec washes with the respective solution minus ^{3}H-digoxin to clear the extracellular space. For each experiment five control ventricles were compared with five experimental ones. Tissues were blotted, weighed, and digested with "NCS" solubilizer obtained from Nuclear-Chicago Corporation and counted in a liquid scintillation counter. External standard quenching correction was employed. Uptake was expressed as disintegrations per minute of ^{3}H-digoxin taken up per milligram of tissue, wet weight. [α-^{32}P]ATP (3 Ci/mmole) was obtained from International Chemical and Nuclear Corporation, Irvine, Calif. $^{35}SO_4$ and ^{3}H-inulin were obtained from New England Nuclear Corporation. The [^{32}P]ATP, $^{35}SO_4$, and ^{3}H-inulin uptake experiments were performed in the same way as the ^{3}H-digoxin experiments but without washing out of the ventricles. Sodium sulfate was added to the $^{35}SO_4$ to give a final concentration of 0.1 mM. ATP was

measured by a modification of the firefly assay for ATP (6).

RESULTS

Hydrolysis of phosphate esters by perfused frog ventricle. The phosphate esters listed in Table 1 were introduced into the perfusion solution of Straub cannulated frog ventricles, and inorganic phosphate present in the perfusate at 5 and 15 min after addition of the ester was determined by the method of Fiske and SubbaRow (5). Further hydrolysis of these substances did not occur once the perfusate was removed from the ventricle, indicating that this hydrolysis was caused by contact of these substrates with the ventricle and was not due to hydrolytic enzymes leaking from the heart. ATP introduced into the perfusate yielded twice as much inorganic phosphate after 5 min of perfusion as did AMP after 5 min of perfusion. The rate of hydrolysis was not linear between 5 and 15 min of perfusion because of substrate depletion. As shown in Table 1, addition of ATP to the perfusate was followed by release of 236 nmoles of inorganic phosphate per 100 mg of tissue in 5 min. This large release cannot have been due simply to the same rate of hydrolysis at each step in the scheme

1. ATP → ADP + P_i
2. ADP → AMP + P_i
3. AMP → adenosine + P_i

since step 3 yielded 122 nmoles of inorganic phosphate per 100 mg of tissue and step 2 released 138 nmoles of P_i per 100 mg of tissue. Since step 2 produced only 16 nmoles more of inorganic phosphate than step 3 in the same time period, most of the 236 nmoles of P_i released from ATP must have come from the complete hydrolysis of ATP to ADP as in step 1, plus subsequent hydrolysis of some of the resulting ADP. It was confirmed that the rate of ATP hydrolysis by the ventricle was far greater than that of ADP or AMP, by measuring the ATP content of the perfusate of a ventricle at intervals after the initiation of perfusion with 0.1 mM ATP. The firefly method of Stanley and Williams (6) was used for this determination. After only 1 min of perfusion more than 66% of the ATP added appeared to be hydrolyzed. A time course of ATP disappearance is shown in Fig. 2. Analysis for ATP in the perfusate from a ventricle perfused for 2 min with solution alone indicated that ATP did not leak into the perfusate from the ventricle, since only 0.6 nM ATP was detected. Because perfusion for 5 min with AMP or ADP resulted in hydrolysis of 61 and 69%, respectively, of the esters added, it was concluded that the rate of ATP hydrolysis was far greater than that of ADP or AMP.

Labeling of ventricles with [α-^{32}P]ATP. Because ATP disappeared rapidly from the perfusion solution, ventricles were labeled for 3

TABLE 1

Hydrolysis of phosphate esters by frog ventricles

The initial concentration of all phosphate esters was 0.1 mM. Each value is the mean ± standard error from perfusion in six ventricles (ventricle weight, 115 ± 6 mg).

Addition to 2 ml of Ringer's solution	P_i released into perfusate	
	5 min	15 min
	nmoles /2 ml/100 mg, wet wt	
None		48 ± 3
β-Glycerol phosphate	86 ± 7	149 ± 7
AMP	122 ± 5	141 ± 5
ADP	138 ± 7	222 ± 9
ATP	236 ± 9	334 ± 9

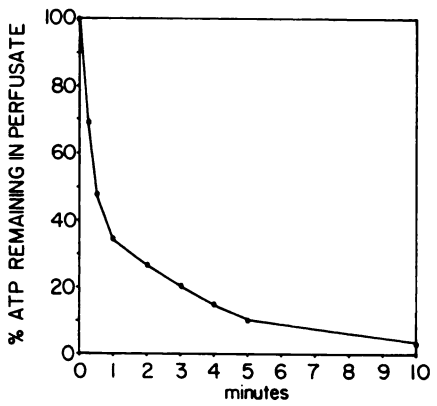

FIG. 2. *Rapid disappearance of ATP added to perfused frog ventricle*

ATP remaining in the perfusate at the times indicated after introduction of 0.1 mM ATP into the 2 ml of perfusate.

min with [α-^{32}P]ATP to determine whether ATP was taken up by the ventricles. ^{32}P spaces determined with [^{32}P]ATP at a concentration of 0.1 mM in three ventricles after 4 min of perfusion were 29.4%, 28.9%, and 29.7%. This was calculated by the formula

$$\left(\frac{\text{cpm/(tissue wt)}}{\text{cpm/(perfusate volume equal to tissue weight)}}\right) \times 100\%$$

where the volume in milliliters is equal to weight in grams of the tissue. For example, if the tissue weight is 100 mg, the ATP space is the ratio of the disintegrations per minute in the tissue to the disintegrations per minute in 100 μl of perfusate $\times$ 100%. Niedergerke (7) has found that the inulin space of ventricles perfused in a manner similar to those reported here was about 18% after 3 min of perfusion while the sulfate space was larger, being about 21%. Experiments in our laboratory with Sephadex-purified, tritiated inulin and with $^{35}SO_4^{2-}$ confirm this. Therefore, on the basis of inulin and $^{35}SO_4^{2-}$ space, a very small percentage of the label from [α-^{32}P]ATP appears to have been taken up intracellularly by the ventricles. This might be in the form of $^{32}P_i$ uptake, since [^{32}P]ATP is rapidly dephosphorylated and the [^{32}P]ADP formed eventually becomes degraded to adenosine and labeled inorganic phosphate. It should be pointed out that in 4 min of perfusion with ATP, 91% of the ATP added, as measured by the firefly method, disappeared from the 2 ml of perfusate (see Fig. 2). From the isotope space studies it can be calculated that only 0.55% of the total amount of ATP hydrolyzed during this time period can be accounted for by intracellular hydrolysis. These findings would seem to exclude the bulk of the intracellular phase as the site of hydrolysis and are consistent with an enzymatic site on or within the cell membrane.

Kinetic properties of p-nitrophenyl phosphate hydrolysis by perfused ventricle. It became apparent that it would be difficult to perform competition studies for the hydrolysis of two different phosphate esters by the perfused ventricle with only inorganic phosphate as an indicator of the reaction. Hence an optical density-monitoring system was developed so that the single selected hydrolysis product could be specifically measured.

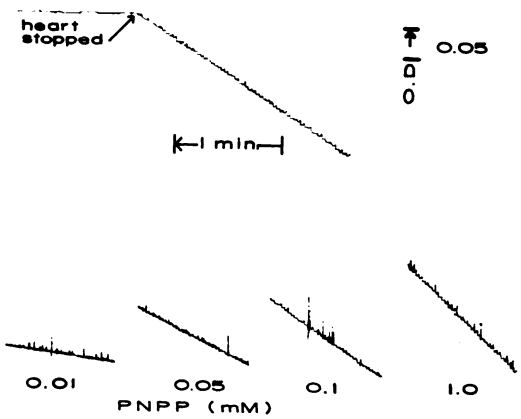

FIG. 3. *Kinetics of p-nitrophenyl phosphate (PNPP) hydrolysis by frog ventricle*

Top: absorbance with respect to time of frog ventricle perfusate monitored spectrophotometrically at 400 mμ after introduction of 0.1 mM *p*-nitrophenyl phosphate into the perfusion solution. Bottom: absorbance at 400 mμ with respect to time after introduction of *p*-nitrophenyl phosphate at the concentrations indicated.

Frog ventricles were mounted in the recording spectrophotometer as described under METHODS. *p*-Nitrophenyl phosphate, when hydrolyzed to *p*-nitrophenol and inorganic phosphate, changes its absorption maximum from 300 mμ to 400 mμ (8). To measure *p*-nitrophenyl phosphate hydrolysis, the spectrophotometer was set at 400 mμ so that increasing absorbance would indicate the production of *p*-nitrophenol, the product of the reaction. In the top tracing of Fig. 3, 0.2 μmole of *p*-nitrophenyl phosphate (final concentration, 0.1 mM) was added to the perfusate of a contracting ventricle mounted in the spectrophotometer. The absorbance immediately began to increase at a constant rate. This change in absorbance stopped abruptly when the stimulator was turned off and the ventricle ceased to contract and to exchange fluid with the cannula. The hydrolytic activity must therefore have been due to contact of the perfusate with the ventricle, and not to

leakage of enzymatic activity into the perfusate. Varying the concentration of *p*-nitrophenyl phosphate from 0.01 to 1 mM gave increasing rates of hydrolysis, as shown in the lower half of Fig. 3. These results, plotted according to Lineweaver and Burk (9), gave straight lines in double-reciprocal plots. The apparent K_m for *p*-nitrophenyl phosphate hydrolysis by the frog ventricle was between 20 and 80 μM in seven preparations studied. As shown in Fig. 4, the addition of *p*-nitrophenyl phosphate to the perfusate of a frog ventricle caused a measurable increase in *p*-nitrophenol appearance during the first two contractions (4 sec) of the ventricle. This rate of hydrolysis continued constant for the next minute, when the wavelength was changed from 400 to 300 mμ to monitor disappearance of substrate from the solution as shown by the decrease in optical density. The rate of decrease in *p*-nitrophenyl phosphate was calculated from this graph, with suitable corrections for *p*-nitrophenol interference at 300 mμ, and was found to equal exactly the appearance of *p*-nitrophenol.

Other phosphate esters were tested as inhibitors of *p*-nitrophenyl phosphate hydrolysis. Table 2 lists these inhibitors and their inhibitory constants. All these agents were shown by double-reciprocal plots to be competitive inhibitors. It is of interest that this enzyme has a greater affinity for the synthetic phosphatase substrate than for the agents listed in the table. An inhibitor constant for ATP inhibition of *p*-nitrophenyl phosphate hydrolysis was not calculated because of the rapid change in ATP concentration when ATP was added to the perfused ventricle. When ATP was added to a ventricle perfused with the substrate, it appeared to be about as potent an inhibitor of *p*-nitrophenyl phosphate hydrolysis as ADP. *p*-Nitrophenyl phosphate hydrolysis (0.1 mM) was not inhibited by 10 μM ouabain or by 28 μM epinephrine.

Validity of coupled enzyme system for measurement of ATP hydrolysis by perfused ventricle. A lactic dehydrogenase–pyruvic kinase coupled enzyme-measuring system was introduced into the perfusion solution for continuous monitoring of the conversion of added ATP to ADP by the frog ventricle.

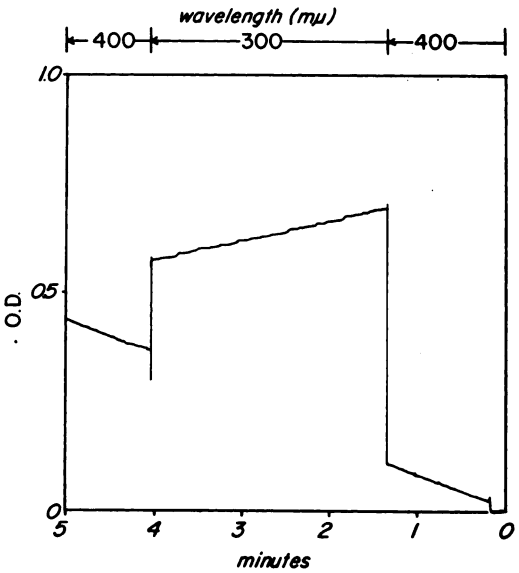

FIG. 4. *Appearance of p-nitrophenol and disappearance of p-nitrophenyl phosphate after addition of substrate to perfused ventricle in the spectrophotometer*

At the beginning of the experiment (bottom right) 2 ml of Ringer's solution containing 1 mM *p*-nitrophenyl phosphate were added and the solution was monitored for the appearance of *p*-nitrophenol at 400 mμ. As indicated, the disappearance of *p*-nitrophenyl phosphate was then monitored at 300 mμ.

This enzyme system consisted of 0.5 mM phosphoenolpyruvic acid, 200 international enzyme units each of pyruvic kinase and lactic dehydrogenase, and 0.325 mM NADH, equivalent to an absorbance of 2.0. This linked enzyme system for measuring (Na^+ + K^+)-ATPase was first suggested by Albers and Koval (10), and its utility has been recently discussed (11). An important advantage of this coupled enzyme-measuring system is that ATP is regenerated, keeping its concentration constant throughout an experiment.

Since this measuring system is a multicomponent system with two coupled reactions, tests were performed to confirm that upon addition of ADP a new steady-state absorbance was reached almost instantaneously. In addition, when the contractions of the heart were stopped in the presence of ATP, the slope of NADH disappearance

TABLE 2

Inhibitors and K_i determined from double-reciprocal plots of p-nitrophenyl phosphate hydrolysis by perfused frog ventricle

p-Nitrophenol production from *p*-nitrophenyl phosphate perfusion of frog ventricles was monitored spectrophotometrically at 400 mμ. The K_m in each experiment was determined from a double-reciprocal plot of the hydrolytic rates at *p*-nitrophenyl phosphate concentrations varied from 0.01 to 1 mM. The inhibitory constants were determined from double-reciprocal plots of *p*-nitrophenyl phosphate hydrolysis at two concentrations of inhibitor (0.1 and 1 mM).

Competitive inhibitor	K_m for *p*-nitrophenyl phosphate	K_i of inhibitor
	mM	*mM*
β-Glycerol phosphate	0.08	0.54
AMP	0.05	0.31
AMP	0.02	0.21
AMP	0.02	0.23
ADP	0.02	0.06
3′-AMP	0.08	0.19
3′-AMP	0.02	0.05
Inorganic phosphate	0.07	1.7

in this system changed instantaneously to zero, also indicating that the measuring system was not lagging and that ATPase enzymes or ADP had not leaked from the heart into the perfusate. Ouabain and epinephrine at the concentrations used in these experiments also had no effect on the coupled enzyme measuring system.

Figure 5 is a tracing from a frog ventricle perfused in the spectrophotometer with Ringer's solution containing 2.0 absorbance units of NADH and the coupled enzyme system. A decrease in absorbance commenced upon the addition of phosphoenolpyruvate. This was due to conversion of phosphoenolpyruvate to pyruvate by the phosphatase activity of the heart. In the presence of pyruvate, the substrate for lactic dehydrogenase, NADH was oxidized and the absorbance decreased. Upon the addition of 10 mM β-glycerol phosphate this hydrolysis stopped. β-Glycerol phosphate and phosphoenolpyruvate were found to be competitive inhibitors of each other, so that

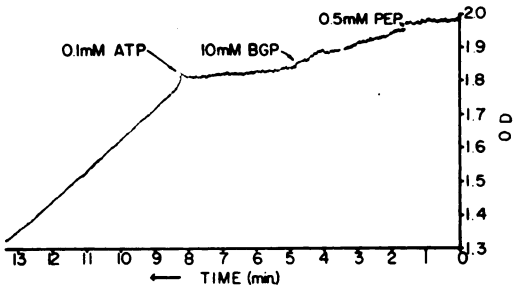

FIG. 5. *Continuous measurement of ATP hydrolysis to ADP by perfused ventricle*

The pyruvic kinase–lactic dehydrogenase-measuring system was included in the perfusate. The decrease in absorbance upon the addition of phosphoenolpyruvate (PEP) indicates its hydrolysis, which was blocked by β-glycerol phosphate (BGP). The subsequent addition of ATP is followed by its rapid conversion by the ventricle to ADP, as indicated by a drop in absorbance or a decrease in NADH.

phosphoenolpyruvate hydrolysis was prevented by adding a large excess of β-glycerol phosphate. In Fig. 5, addition of 0.1 mM ATP caused a sharp and immediate decline in optical density, indicating that the heart was rapidly hydrolyzing the added ATP. The rate was constant during the course of the experiment. In other experiments the concentration of ATP was varied, a double-reciprocal plot was constructed, and the apparent K_m for ATP hydrolysis was found to be about 0.1 mM.

Correlation of inhibition of ATPase in frog ventricle by ouabain with positive inotropic effect. Figure 6 shows the effect of ouabain on ATP hydrolysis in a typical frog ventricle. The initial decline in absorbance was due to hydrolysis of ATP after addition of the complete ADP analytical measuring system, including 10 mM β-glycerol phosphate and 0.1 mM ATP, as was done in the experiments shown in Fig. 5. The upper graph indicates the stroke volume of the ventricle, corresponding to the elapsed time. At the point indicated, 3 μM ouabain was added and perfusion was continued. The stroke volume of the ventricles increased at the same time that a decline in slope of the absorbance time curve was observed. This concentration of ouabain is

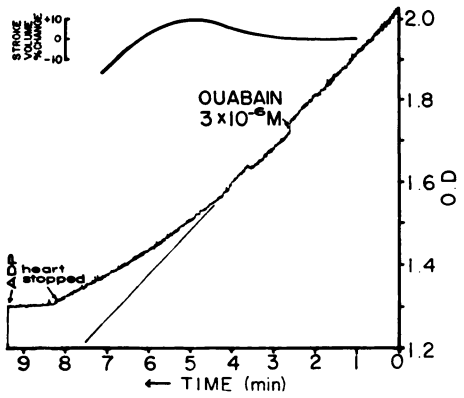

FIG. 6. *Correlation of inhibition of ATPase by ouabain with positive inotropic effect*

The main tracing shows change in absorbance at 340 mμ of the perfusate from a 78-mg frog ventricle containing the ADP-measuring system, with respect to time. At the arrow, ouabain was added in a volume of 10 μl to make the 2-ml perfusate concentration 3 μM with respect to ouabain. At top of figure are shown the stroke volume changes after addition of ouabain, obtained simultaneously from the plethysmographic transducer.

eventually toxic to frog hearts, as indicated by the later decline in stroke volume. In Fig. 6 the stimulator was turned off at 8.2 min thus immediately isolating the perfusate from contact with the ventricle. The slope of ATP hydrolysis immediately became zero, indicating that the measuring system was not inhibited or lagging. ADP was then added, and the slope abruptly changed, indicating that the measuring system was not inhibited but that the ATPase activity of the ventricle was inhibited. Furthermore, these records show that inhibition of the ATPase began well before the onset of toxicity and was coincident with the positive inotropic effect of the glycoside. Besch *et al.* (12) have recently shown in a different kind of experiment that $(Na^+ + K^+)$-ATPase prepared from hearts treated with inotropic amounts of ouabain was inhibited.

Potentiation of digoxin toxicity by ATP in perfused frog ventricle. Ventricles mounted on ordinary Straub cannulae were perfused with Ringer's solution, and the stroke volume was recorded as described under METHODS. In this study, a 50% decrease in stroke volume from control levels was chosen as a convenient index of toxicity. In four ventricles without ATP, 3 μM digoxin caused a 50% decrease in stroke volume in 18 min (SEM = ±1.0 min). In another four ventricles treated with digoxin plus 0.1 mM ATP, a 50% decrease in stroke volume occurred in 11.8 min (SEM = ± 1.0 min) ($p < 0.05$). As a control, perfusion in three ventricles with the above concentration of ATP for 15 min caused only a 2% ± 6% (SEM) decrease in stroke volume. It thus appears that ATP enhances the pharmacological effect of digoxin.

TABLE 3

Effect of ATP, AMP, and epinephrine on ^{3}H-digoxin uptake by frog ventricle

Ventricles were perfused as indicated for 3 min with ^{3}H-digoxin in Ringer's solution and ATP, AMP, or epinephrine as indicated. The hearts were then given three 30-sec washes, blotted, weighed, digested, counted by liquid scintillation, and corrected for quenching. Statistical significance refers to Student's *t*-test.

Treatment	^{3}H-Digoxin uptake[a]	No. of ventricles	*p* vs. control
	dpm/mg, wet wt		
Experiment A			
3 μM ^{3}H-digoxin	214 ± 9	5	
3 μM ^{3}H-digoxin + 0.1 mM ATP	328 ± 33	5	<0.005
Experiment B			
3 μM ^{3}H-digoxin	207 ± 16	5	
3 μM ^{3}H-digoxin + 0.1 mM AMP	202 ± 9	5	NS[b]
Experiment C			
3 μM ^{3}H-digoxin	254 ± 12		
3 μM ^{3}H-digoxin + 25 nM epinephrine	276 ± 9	5	NS
Experiment D			
3 μM ^{3}H-digoxin	247 ± 12	5	
3 μM ^{3}H-digoxin + 2.5 μM epinephrine	238 ± 22	5	NS

[a] Results are means ± standard errors

[b] Not significant.

^{3}H-Digoxin uptake by perfused frog ventricle. Since ATP seemed to increase the potency of digoxin in the perfused ventricle,

the possibility that the responsible mechanism might be an increased rate of uptake by the heart was tested. Matsui and Schwartz (13) have shown that ATP increases cardiac glycoside binding to the isolated ($Na^+ + K^+$)-sensitive ATPase *in vitro*. Ventricles were prepared and labeled for 3 min as described under METHODS. The addition of 0.1 mM ATP significantly increased the uptake of ^{3}H-digoxin in the perfused ventricle, as shown in Table 3, experiment A. Five control ventricles were included on the same day with each set of five experimental ventricles. Since ATP is positively inotropic in the frog ventricle (14), another inotropic agent, epinephrine, was also tested to determine whether the increased uptake of ^{3}H-digoxin in the presence of ATP was due to the augmentation of contractility by ATP. As shown in experiments C and D, Table 3, epinephrine did not alter ^{3}H-digoxin uptake at two inotropic concentrations. Since inotropic activity did not alter digoxin binding in the frog ventricle, AMP was tested to determine whether the presence of a nucleotide other than ATP could alter the uptake of ^{3}H-digoxin. The results in Table 3, experiment B, show that AMP had no effect on digoxin uptake in the frog ventricle, in contrast to the large increase in uptake in the presence of ATP. It was thus concluded that ATP could increase the pharmacological properties of digoxin in the frog ventricle by increasing binding of the glycoside, presumably to the ventricular ($Na^+ + K^+$)-ATPase, if extrapolation from work *in vitro* is valid.

DISCUSSION

The intact, cannulated frog ventricle was chosen for these experiments because it would make interpretation of the enzymatic localization less difficult. Cutting of tissue is not necessary in this preparation, so that enzymatic activity at cut muscle fiber surfaces need not be considered. Also, since the frog heart contains no vascular system, the experimental perfusion system for these ventricles is much the same as *in vivo*, where the blood that is pumped also bathes the muscle fibers. Finally, the muscle fibers of the frog ventricle contains no transverse tubular system (15), so that penetration of substrate by this route is not possible.

Strong evidence for a membrane-localized ATPase is the finding that [^{32}P]ATP did not enter the muscle fibers to any appreciable extent during a period when 91% of the added ATP was hydrolyzed. A [^{32}P]ATP space of 30% could account for only 0.55% of the hydrolyzed ATP, assuming that 20% is the true extracellular volume. Since the ratio of the 2-ml perfusate volume to ventricular cell volume (100 mg) is about 20:1, if the hydrolyzed ATP were distributed equally between the ventricle and perfusate 4.6% should have been found in the ventricle. Since only about one-tenth this amount was found, it appears likely that ATP is not hydrolyzed intracellularly. However, hydrolysis in a small, rapidly turning over membrane pool of less than 10% of the tissue volume cannot be ruled out by these experiments. In further support of a membrane-localized ATPase is the finding that when production of ADP was measured in the perfusate the inorganic phosphate released equaled the ADP produced (calculated from Table 1 and Fig. 6). The exact location of this apparent membrane ATPase activity must await more detailed kinetic and histological studies. The finding that ATP does not penetrate the myocardium intact was first reported by Hoffmann and Okita (16) in the guinea pig heart. Although other workers (17, 18) have suggested that ATP penetrates the myocardium, their data do not preclude its prior dephosphorylation, as suggested by Hoffmann and Okita.

The results presented in this paper show that ouabain inhibits an ATPase activity of the frog ventricle coincident with the positive inotropic effect of the drug. It appears in many ways to resemble the ($Na^+ + K^+$)-ATPase isolated from heart muscle, since it is inhibited by ouabain, seems to be localized in the membrane, and takes up labeled glycoside in the presence of ATP but not AMP. Besch *et al.* (12) have shown that ($Na^+ + K^+$)-ATPase prepared from dog hearts is inhibited during the positive inotropic influence of ouabain.

The phenyl phosphatase in the ventricle

was not inhibited by ouabain, even at very high concentrations. It is commonly assumed that *p*-nitrophenyl phosphate is an artificial substrate (19) for the potassium-stimulated phosphatase which dephosphorylates the phosphate-protein intermediate created by the sodium-stimulated kinase of the $(Na^+ + K^+)$-ATPase system. It is generally thought that cardiac glycosides act at this second step, thereby limiting the dephosphorylation of the ATPase enzyme. Two explanations of these findings are possible: either (a) the frog ventricle *p*-nitrophenyl phosphatase is not a part of the $(Na^+ + K^+)$-ATPase system or (b) it is a part of the system, but inhibition by ouabain is not apparent for other reasons. *p*-Nitrophenyl phosphate might not be as good a substrate as the true phosphoprotein undoubtedly present in this intact, beating preparation. In the presence of the true substrate, the artificial substrate might be ineffective. In fact, it is thought that the cardiac glycosides inhibit dephosphorylation of the phosphorylated intermediate by binding to it (13). It seems unlikely that ouabain would bind to *p*-nitrophenyl phosphate. Another reason for lack of phosphatase inhibition might be the presence of sodium in the perfusate, which has been reported to inhibit this enzyme (20). Inhibition by sodium ion might reduce the effectiveness of ouabain as an inhibitor of the phosphatase.

The cardiotoxicity of digoxin is increased in the frog ventricle in the presence of ATP. In addition, ATP added to the perfusate of the ventricles significantly increased the binding of labeled digoxin to the ventricle. The increased toxicity and uptake of the drug would be expected if it were acting upon the $(Na^+ + K^+)$-ATPase. The inhibition of the ATPase in the frog ventricle appears to increase with time after addition of the drug. One explanation for this might be that the uptake of digoxin by the $(Na^+ + K^+)$-ATPase is not instantaneous. In the presence of Mg^{2+}, ATP, and Na^+, in another study (21), the half-time for complete binding of tritiated digoxin or ouabain to the isolated $(Na^+ + K^+)$-ATPase was about 15 min. Matsui and Schwartz (13) have shown that ATP, but not AMP, caused binding of digoxin to the purified $(Na^+ + K^+)$-ATPase isolated from cardiac muscle. Because ATP is positively inotropic in the frog ventricle, epinephrine was used in the present uptake studies as a control for inotropic activity. Both concentrations of epinephrine failed to alter the uptake of the drug into the ventricles. Recently it has been shown that inotropic activity does not alter cardiac glycoside uptake in the perfused guinea pig atrium (22). Since tritiated digoxin was taken up by the ventricles without ATP in the perfusate, it is possible that endogenous membrane pools of ATP exist which are utilized for the binding of the drug to the receptor. If this is true, it is conceivable that patients refractory to the drug may have some deficiency in their myocardial membrane stores of ATP or of the $(Na^+ + K^+)$-ATPase system.

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