MOLECULAR PHARMACOLOGY, 20:551-557

Interaction of Chlormadinone Acetate with the Ouabain Binding Site of Na^+,K^+-ATPase

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Received December 8, 1980; Accepted June 16, 1981

SUMMARY

Chlormadinone acetate, a hydroxyprogesterone derivative, reversibly inhibits both Na^+,K^+-ATPase activity and [^{3}H]ouabain binding to the enzyme. Chlormadinone acetate substitutes for ouabain in "chasing" the label from the enzyme-[^{3}H]ouabain complex. Chlormadinone acetate is a noncompetitive inhibitor of Na^+,K^+-ATPase with respect to ATP and is a competitive inhibitor with respect to potassium. The monovalent cation site which regulates ouabain binding to Na^+,K^+-ATPase (apparent K_D for potassium = 1.4 mM) also regulates chlormadinone binding to Na^+,K^+-ATPase (apparent K_D = 0.96 mM). The I_{50} values for chlormadinone acetate inhibition of Na^+,K^+-ATPase isolated from sheep kidney, cat heart, and guinea pig heart were 3, 6, and 30 μM, respectively. Similar differences in species sensitivity have been found for ouabain and ouabagenin. These data suggest that chlormadinone acetate binds to the ouabain binding site of isolated Na^+,K^+-ATPase. Concentrations of chlormadinone acetate up to 30 μM can be obtained in unstirred solutions in glass vessels. However, the highest concentrations that could be obtained in muscle baths containing isolated strips of guinea pig left atria, or strips of cat atria and ventricle, aerated with 95% O_2-5% CO_2 were only 0.5–4 μM, and these concentrations did not prevent ouabain from binding to its receptor in intact muscles. Thus, it appears that chlormadinone acetate is not capable of modifying the pharmacological action of ouabain or of producing an inotropic action independently under these experimental conditions because of its limited solubility.

INTRODUCTION

It is commonly accepted that Na^+,K^+-ATPase (EC 3.6.13) is the membrane-bound enzyme system responsible for the active transport of Na^+ and K^+ across cell membranes (1). Cardiac glycosides specifically inhibit this enzyme system. Since the first demonstration of specific binding of [^{3}H]digoxin and [^{3}H]ouabain to membranes enriched in Na^+,K^+-ATPase (2), and modulation of the binding by sodium and potassium, evidence has accumulated that suggest that the Na^+,K^+-ATPase is the pharmacological receptor for digitalis (3). A high correlation between the positive inotropic effect on hearts of guinea pigs (4), dogs (5), and cats (6) and the occupation of ouabain binding sites on Na^+,K^+-ATPase isolated from the same hearts has been demonstrated. These and other related studies suggest that the positive inotropic effect is mediated by the binding of ouabain to Na^+,K^+-ATPase and possible subsequent inhibition of enzyme activity.

Recently, it was shown that chlormadinone acetate, a hydroxyprogesterone derivative, inhibited Na^+,K^+-ATPase in microsomes prepared from guinea pig brain and reduced [^{3}H]ouabain binding to a Na^+,K^+-ATPase-containing fraction prepared from dog ventricle (7). Chlormadinone acetate also affected internal sodium content and sugar transport in guinea pig atria but did not cause a positive inotropic effect in guinea pig heart (8). On the basis of these results it was suggested that (a) chlormadinone acetate binds to the ouabain site on the Na^+,K^+-ATPase; (b) sodium pump inhibition by chlormadinone acetate or cardiac glycosides does not necessarily lead to increased contractility; and (c) Na^+,K^+-ATPase may not be the receptor responsible for the inotropic effect of cardiac glycosides.

This study was supported by Grants PO1 HL-22619-03 (Mission I) and T32 HL-07382-04 from the United States Public Health Service, National Institutes of Health, and by the American Heart Association, Southwest Ohio Chapter.

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0026-895X/81/030551-07$02.00/0

The ability of a compound to prevent [^{3}H]ouabain binding to Na^+,K^+-ATPase is necessary but not sufficient proof that the compound interacts at the same physical site as does ouabain. There are many compounds, such as irreversible inhibitors like *N*-ethylmaleimide (9, 10), detergent-like compounds such as palmitylcarnitine (11, 12), alkaloids such as sanguinarine (13), and even potassium (14), which under appropriate conditions inhibit enzyme activity and [^{3}H]ouabain binding to the Na^+,K^+-ATPase. It is unlikely that these agents bind to the same physical site as ouabain. Because the preliminary data published thus far using chlormadinone acetate (8) appear to cast some doubt on the concept that Na^+,K^+-ATPase is the pharmacological receptor for digitalis, further experiments to characterize the interaction of chlormadinone acetate with Na^+,K^+-ATPase and to investigate the lack of a positive inotropic effect on isolated cardiac tissues were carried out.

EXPERIMENTAL PROCEDURES

Na^+,K^+-ATPase, purified from the outer medulla of lamb kidney (15), was kindly supplied by Dr. L. K. Lane, University of Cincinnati College of Medicine, Cincinnati, Ohio. The procedure yielded an enzyme with a specific activity of 1 mmole of P_i/hr/mg of protein. Na^+,K^+-ATPase was also partially purified from cat, guinea pig, and rat heart as previously described (16) with a specific activity of 0.02–0.03 mmole of P_i/hr/mg of protein. The ouabain-sensitive fraction was 80–85% of the total activity for the cardiac preparations and 99% for the kidney preparation. Protein was estimated by the method of Lowry *et al.* (17). Na^+,K^+-ATPase activity was determined at 37° using a spectrophotometric linked-enzyme assay (18). The medium contained 25 mM histidine (pH 7.2), 5 mM Na_2ATP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0.4 mM NADH, 1 mM phosphoenolpyruvate, and 20 μl of pyruvate kinase/lactate dehydrogenase suspension (Sigma Chemical Company, St. Louis, Mo.). Since this suspension contained large quantities of ammonium ion, this assay was not suitable for studies in which the concentration of potassium was varied. Instead, a colorimetric assay for the released P_i as described by Fiske and SubbaRow (19) was used. The reaction was carried out in a medium containing 100 mM NaCl, 25 mM histidine (pH 7.4), 5 mM Na_2ATP, 5 mM $MgCl_2$, and various concentrations of potassium.

Tritium-labeled ouabain (New England Nuclear Corporation, Boston, Mass.) was diluted with unlabeled ouabain to a specific radioactivity of 100 mCi/mmole. Binding of [^{3}H]ouabain to the purified Na^+,K^+-ATPase was determined in the presence of 5 mM $MgCl_2$, 5 mM Tris-phosphate, and 50 mM Tris HCl (pH 7.4) at 37°. At appropriate times, 20-μg enzyme aliquots were removed, filtered through 0.22-μm Millipore filters, and washed three times with 5 ml of cold distilled water. The filters were placed in scintillation fluid (Aquasol II, New England Nuclear Corporation), and radioactivity was measured in a Beckman LS 8100 liquid scintillation counter.

Chlormadinone acetate was dissolved in ethanol to a final concentration of 10^{-2} M. The ethanol concentration in all assays was less than 1%. Both the [^{3}H]ouabain binding and the Na^+,K^+-ATPase assays were corrected for the slight (<5%) inhibitory effect of ethanol.

Guinea pigs or cats received simultaneous injections of sodium pentobarbital (30 mg/kg i.p.) and 2500 units of heparin. Each animal was ventilated with 95% O_2-5% CO_2. The heart was then removed through a left thoracotomy and perfused with modified Krebs-Henseleit bicarbonate buffer containing (millimolar concentrations) NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; Na_2EDTA, 0.5; $NaHCO_3$, 25; glucose, 11; and heparin, 5 units/ml. During perfusion, either a right ventricular papillary muscle or a strip from the right ventricle about 5 mm long, 0.5–1.0 mm thick, and 2 mm wide and an atrial strip of the same dimensions were excised. One end of the muscle strip was fixed with a 5-0 monofilament surgical suture to a Grass FTO3C force displacement transducer. The other end was clamped to a bipolar, punctate platinum electrode affixed to a stainless steel rod. The muscle preparation was placed in a muscle bath (50-ml volume) containing modified Krebs-Henseleit bicarbonate solution bubbled with 95% O_2-5% CO_2. The temperature of the bath was kept at 37 ± 0.5°. Electrical stimulation was applied from a Grass S4 stimulator through two platinum electrodes fixed to the clip holding the muscle. Stimulation was at 1.5–2.0/sec for 1 msec at a voltage 15% above threshold. The isometric force of contraction and its first derivative were recorded on a Grass Polygraph P7. The muscles were equilibrated for 1 hr with a 1-*g* preload and then tested for their response to $CaCl_2$ (0.5 mM for 2 min). Five washouts were then carried out. After 10 min the tension was measured and taken as control tension. The bath solution (50 ml) was removed and replaced with sonicated Krebs-Henseleit solution or chlormadinone acetate (10 nmoles/ml) (actual concentration 1.1 μM) added to the Krebs-Henseleit solution. The response to ouabain in the presence of chlormadinone acetate was determined by exposing strips to added chlormadinone acetate (50 nmoles/ml) (actual concentration 3.1 μM) followed at 20-minute intervals with increasing concentrations of ouabain (20, 50, 100, and 200 nM).

To titrate the number of Na^+,K^+-ATPase sites occupied, the procedure of Michael *et al.* (6) was followed. Papillary muscles were placed in a muscle bath as described above. After treatment, the muscles were removed, blotted, and frozen on dry ice. At a convenient time, each muscle was thawed and homogenized with a Polytron (Brinkman Instruments) in the cold, five times for 20 sec with 5-sec intervals for cooling, in 2–4 ml of a medium containing in final concentration, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl and 2.5 mM $MgCl_2$. This homogenate was diluted with the above medium to a concentration equivalent to 10 mg of tissue (wet weight) per milliliter. Aliquots of this homogenate were added to a solution containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2.5 mM Na_2ATP, 2.5 mM $MgCl_2$, and 10 nM [^{3}H] ouabain (14.4 Ci/mmole). The assay medium contained 0.15–0.25 mg of protein in 2 ml. At appropriate times, 0.4-ml aliquots were taken and filtered through 0.22-μm Millipore filters and rinsed three times with 2 ml of cold water. Radioactivity was determined as described above.

RESULTS

Chlormadinone acetate is a reversible inhibitor of purified lamb kidney Na^+,K^+-ATPase, since the percentage inhibition did not change with time. Consistent with this result, a 100-fold dilution of a chlormadinone acetate-inhibited enzyme completely restored enzyme activity in less than 30 sec (data not shown). A Scatchard-type analysis of the inhibition data indicated that the interaction of chlormadinone acetate with the enzyme was at a single class of sites having under these conditions (100 mM NaCl, 10 mM KCl) an apparent dissociation constant (K_i) of 3.7 μM (Fig. 1). Chlormadinone acetate thus resembles the easily reversible inhibitor, ouabagenin, more than it does ouabain, which binds to Na^+,K^+-ATPase in a pseudo-irreversible manner (20). Consistent with the work of Chow *et al.* (7), who used a crude Na^+,K^+-ATPase preparation, chlormadinone acetate also inhibited [^{3}H]ouabain binding to a purified Na^+,K^+-ATPase in a time and concentration-dependent manner (Fig. 2). Since the affinity of the enzyme for ouabain is 100-fold higher than the affinity of the enzyme for chlormadinone acetate, [^{3}H]ouabain binding at intermediate concentrations of chlormadinone acetate tended to reach the same levels as in the absence of chlormadinone acetate if sufficient time was allowed. These results are similar to those described for the effect of ouabagenin on [^{3}H]ouabain binding (20).

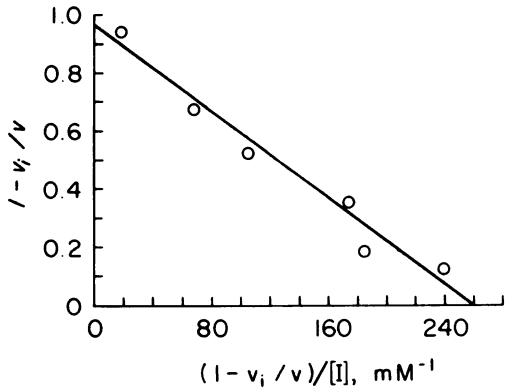

FIG. 1. *Scatchard analysis of the inhibition of lamb kidney Na^+,K^+-ATPase by chlormadinone acetate*

v, Enzyme activity in the absence of inhibitor; v_i, enzyme activity in the presence of inhibitor at concentration [I]. The *line* represents the result of a least-squares fit. The dissociation constant ± standard deviation was 3.71 ± 0.36 μM.

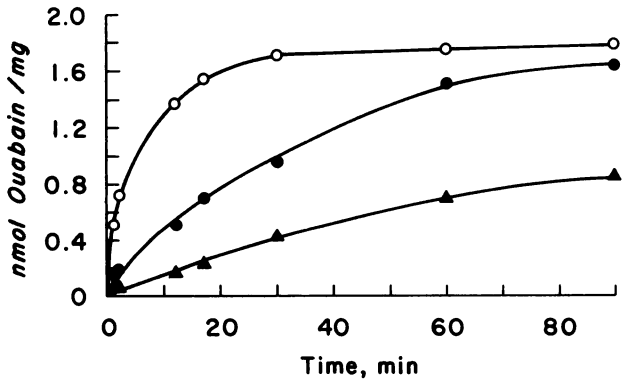

FIG. 2. *Effect of chlormadinone acetate on [^{3}H]ouabain binding*

Time course of [^{3}H]ouabain (0.25 μM) binding at 37° in the presence of 5 mM $MgCl_2$, 5 mM Tris-phosphate, and 50 mM Tris-HCl (pH 7.4) to Na^+,K^+-ATPase purified from lamb kidney. The concentrations of chlormadinone acetate were zero (○), 2 μM (●), and 10 μM (▲). The enzyme concentration was 20 μg/ml.

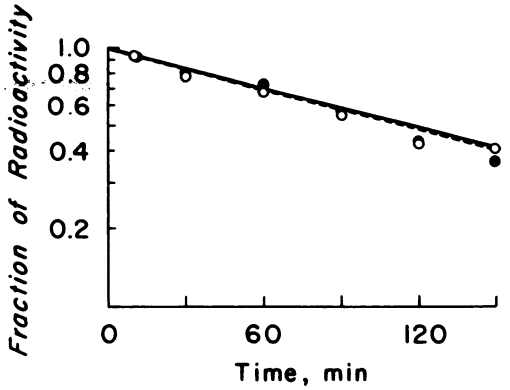

FIG. 3. *Effect of chlormadinone acetate on dissociation of ouabain from lamb kidney Na^+,K^+-ATPase at 37°*

Enzyme-[^{3}H]ouabain complex was formed as indicated under Experimental Procedures. At time zero, chlormadinone acetate (○) or ouabain (●) was added, each in an amount that would yield a final concentration of 50 μM. At the times indicated, aliquots were removed, filtered, and washed. A typical experiment is shown. Means (± standard error of the mean) of six experiments yielded half-times of 110 ± 6 min for ouabain and 118 ± 8 min for chlormadinone acetate.

To determine whether chlormadinone acetate is a specific or a nonspecific inhibitor of enzyme activity and ouabain binding, the ability of ouabain and chlormadinone acetate to chase [^{3}H]ouabain from its binding site on the enzyme was investigated (Fig. 3). The half-times for the dissociation at 37° were almost identical (100 min for ouabain and 118 min for chlormadinone acetate). These data are consistent with the hypothesis that chlormadinone acetate is acting at the ouabain site of the enzyme but certainly does not prove this hypothesis. Any agent which can prevent ouabain binding without damaging the enzyme would yield the same results.

It is known that the rate of association of ouabain to the enzyme and consequently the affinity of the enzyme for ouabain depends upon the presence and concentration of certain physiological ligands (21). In particular, the affinity of the enzyme for ouabagenin decreases sharply with increasing concentrations of potassium up to 5 mM and then levels off (20). Similar results were obtained with chlormadinone acetate analyzed by a Hunter-Downs plot (22), which showed apparent competitive inhibition by chlormadinone acetate with respect to potassium up to 4 mM and noncompetitive inhibition at higher concentrations (Fig. 4). By extrapolating to zero potassium, the K_i for chlormadinone acetate is 0.6 μM in the absence of potassium. At saturating potassium (6–10 mM), the K_i is 3–4 μM. Therefore, potassium decreases the apparent affinity of the enzyme for chlormadinone acetate as it does for ouabagenin (20) and for ouabain (14, 21). We have suggested that a monovalent cation site regulates ouabain binding to Na^+,K^+-ATPase (14). In the presence of saturating magnesium, ATP, and sodium, the K_D of this site for potassium is 1.4 mM as determined by ouabain binding to Na^+,K^+-ATPase pre-

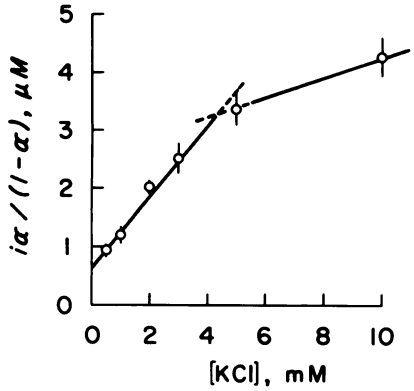

FIG. 4. *Hunter-Downs plot: the effect of potassium on the ability of chlormadinone acetate to inhibit lamb kidney Na^+,K^+-ATPase*

$\alpha = v_i/v$, where v_i and v are velocities in the presence and absence of chlormadinone acetate, respectively. The concentrations, i, of chlormadinone acetate were 0.5, 1, 3, and 10 μM. Na^+,K^+-ATPase activity was measured in a medium containing 100 mM NaCl, 25 mM histidine (pH 7.1), 5 mM Na_2ATP, 5 mM $MgCl_2$, and concentrations of potassium as shown. Inorganic phosphate was determined by the method of Fiske and SubbaRow (19). The results shown are the means (± standard error of the mean) of four to nine determinations at each KCl concentration. The intercept on the ordinate is the apparent dissociation constant for chlormadinone acetate (0.6 μM) in the absence of potassium.

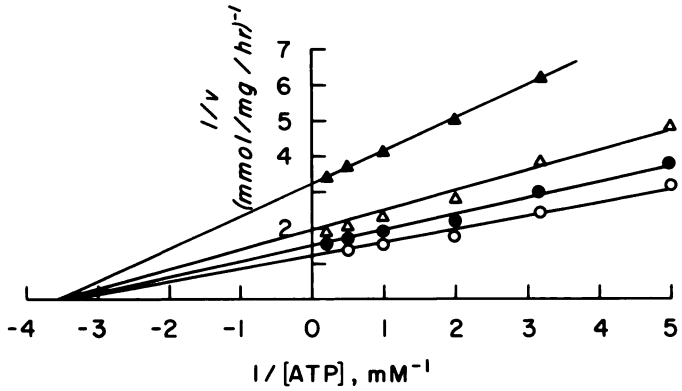

FIG. 5. *The effect of ATP on inhibition of enzyme activity by chlormadinone acetate*

Enzyme activity, v, at 37° in the presence of 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 25 mM histidine (pH 7.4) was determined using a spectrophotometric method described under Experimental Procedures. The concentrations of chlormadinone acetate were zero (○), 1 (●), 3 (△), and 10 μM (▲). Average values for two experiments are shown. The *lines* were obtained by direct-fitting to $v = v^i_{max}/(1 + K_m/S)$, where v^i_{max} is maximal activity at each concentration of inhibitor, S is concentration of ATP, and K_m (0.27–0.35 mM) is the dissociation constant for ATP.

pared from lamb kidney (18). A K_D for potassium of 0.96 mM was calculated from the data presented in Fig. 4.

The inhibition of Na^+,K^+-ATPase by chlormadinone acetate with respect to ATP was examined (Fig. 5). The Lineweaver-Burk analysis indicated noncompetitive inhibition of Na^+,K^+-ATPase activity with respect to ATP. Chlormadinone acetate, like ouabagenin (20), did not affect the affinity of the enzyme for ATP (K_m for ATP = 0.27–0.35 mM) but caused a reduction in the apparent V_{max}. Replots of the $1/v$ intercepts of Fig. 5 versus concentration of chlormadinone acetate were linear, consistent with simple noncompetitive inhibition with respect to ATP, and yielded a K_i for chlormadinone acetate under these conditions of 6 μM. The regulation by ligands of interaction of chlormadinone acetate with Na^+,K^+-ATPase is identical with that of cardiac glycosides. The sensitivities of Na^+,K^+-ATPase from various species to inhibition by chlormadinone acetate and ouabagenin show similar trends (Fig. 6). The I_{50} values of chlormadinone acetate for inhibition of enzyme isolated from sheep kidney, cat heart, and guinea pig heart are 3, 6, 30 μM, respectively. Chlormadinone acetate at concentrations up to 100 μM did not inhibit enzyme prepared from rat heart or kidney. Similar differences in species sensitivity are found for ouabain and ouabagenin (23). Because of their greater solubility and higher affinity, high concentrations of ouabain and ouabagenin have been shown to inhibit Na^+,K^+-ATPase prepared from rat heart and kidney.

Chlormadinone acetate did not affect the contractility of atria of ventricular strips of guinea pig, consistent with earlier reports (8). It also did not affect the contractility of cat atria or ventricle (Fig. 7A), which should be somewhat more sensitive to chlormadinone acetate (Fig. 6). Chlormadinone acetate did not prevent or antagonize the positive inotropic action of ouabain (Fig. 7B).

Since inhibition of Na^+,K^+-ATPase prepared from guinea pig heart appeared to plateau at an apparent chlormadinone acetate concentration of 30 μM (Fig. 6), the solubility of chlormadinone acetate was examined (Fig. 8). Chlormadinone acetate had an absorbance maximum at 282 nm and at concentrations up to 10 μM followed Lambert-Beer's law, showing a proportional increase in absorbance with concentration. The absorbance reached a maximum at approximately 30 μM. Attempts to increase the concentration by adding more chlormadinone acetate resulted in precipitation, and the absorbance (after centrifugation to remove insoluble material) decreased (Fig. 8). Interestingly, the absorbance of solutions of chlormadinone acetate used in the muscle baths after 10 min of aerating with 95% O_2-5% CO_2 was only 0.025–0.060 regardless of the amount of chlormadinone acetate added to the bath. The actual free concentration of chlormadinone acetate in the muscle bath thus appears to be 0.5–4 μM, which is 7 to 60-fold lower than

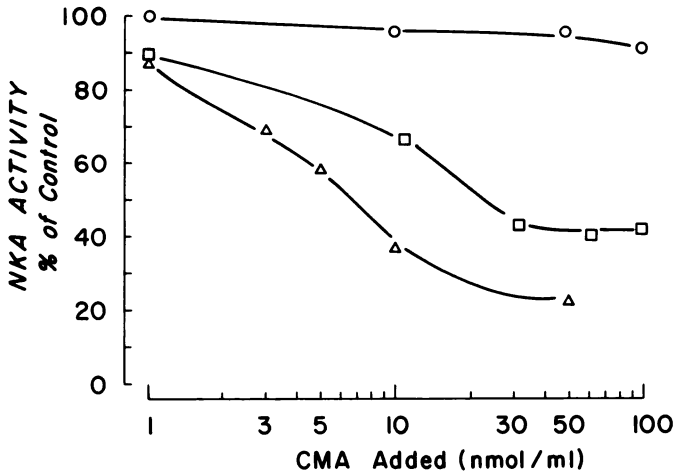

FIG. 6. *The Na^+,K^+-ATPase activity of cat (△), guinea pig (□), and rat (○) heart enzyme measured spectrophotometrically versus the amount of chlormadinone acetate (CMA) added to the cuvette*

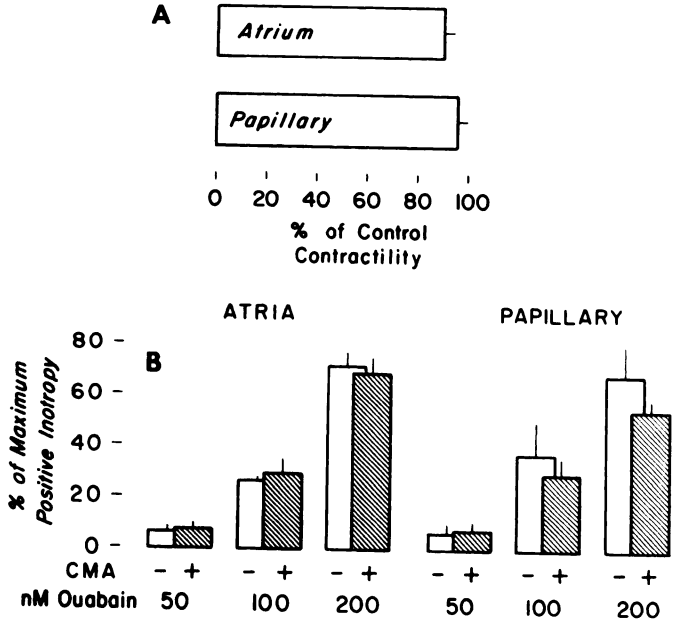

FIG. 7. *Cat atrial and papillary muscle response to chlormadinone acetate (CMA)*

A. The effect of added chlormadinone acetate (10 nmoles/ml) (actual concentration averaged 1.1 μM) on contractile force developed by cat atrial and papillary muscle strips is expressed as percentage of developed force in the absence of chlormadinone acetate (control = 100%). B. The developed force response of a cat atrial and papillary muscle strips when exposed to increasing concentrations of ouabain (50, 100, and 200 nM) in the presence (*hatched bars*) and absence (*open bars*) of added chlormadinone acetate (50 nmoles/ml) (actual concentration averaged 3.1 μM).

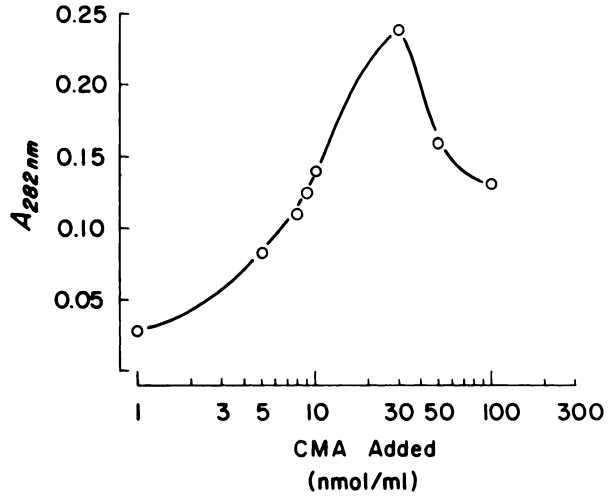

FIG. 8. *Solubility of chlormadinone acetate (CMA)*

Chlormadinone acetate was added to water and centrifuged for 30 min at 100,000 × *g* (37°). Absorbance of supernatant was measured (○). Identical results were obtained in Krebs-Henseleit solution.

the concentration which could be obtained in unstirred solutions in glass containers. Since at this free concentration one would predict from studies on isolated enzyme 0–35% inhibition (Fig. 6) and since chlormadinone acetate neither acted as an agonist nor antagonized the positive inotropic effect of ouabain in isolated heart studies, we attempted to determine whether chlormadinone acetate actually interacted with the Na⁺,K⁺-ATPase of intact muscles under the conditions of the contractility measurements, using the procedure of Michael *et al.* (6). Homogenates from cat papillary muscles which were not treated with any drug bound at equilibrium an average (± standard error of the mean) of 2.2 ± 0.1 picomoles of [³H]ouabain per milligram of protein (Fig. 9). Exposure of muscle to 0.1 μM unlabeled ouabain for 15 min in the muscle bath produced a positive inotropic effect. Homogenates from these muscles bound at equilibrium 1.4 ± 0.3 picomoles of [³H]ouabain per milligram of protein. Thus the 0.1 μM unlabeled ouabain in the muscle bath occupied 36% of the receptors during the 15-min exposure. After exposure of muscles to 3 μM ouabagenin for 15 min, 0.1 μM unlabeled ouabain was added to the bath for an additional 15 min. Binding of [³H]ouabain to homogenates of these muscles was reduced only by 10%. These results indicate that in the muscle bath the high concentration of ouabagenin partially prevented the unlabeled ouabain from binding to its receptors. In contrast, homogenates from muscles exposed to added chlormadinone acetate, 10 nmoles/ml (actual concentration 1.1 μM) for 15 min followed by 0.1 μM unlabeled ouabain for 15 min bound 1.3 ± 0.1 picomoles of [³H]ouabain per milligram of protein (Fig. 9), indicating that chlormadinone acetate did not prevent the unlabeled ouabain in the muscle bath from binding to the Na⁺,K⁺-ATPase of the muscles. In comparison, 2 μM chlormadinone acetate decreased the binding of 0.25 μM [³H]ouabain at 15 min to isolated enzyme by 60% (Fig. 2). These data indicate that, under the conditions of our experiments, chlormadinone acetate at the concentrations used in these experiments does not react with the Na⁺,K⁺-ATPase of intact muscles, presumably because the concentration of the drug at the receptor is too low.

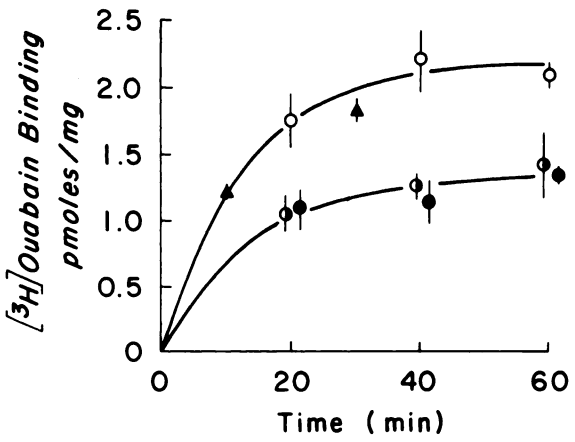

FIG. 9. *[³H]ouabain binding to homogenates of cat papillary muscles*

Cat papillary muscles were exposed to chlormadinone acetate (●), 10 nmoles/ml (actual concentration, 1.1 μM), 3 μM ouabagenin (▲), or neither compound (◑) in a muscle bath for 15 min. Ouabain (0.1 μM) was then added for an additional 15 min. Controls (○) were muscles that were not treated with any drug. The muscles were removed and homogenates were prepared. [³H]ouabain was then bound to unoccupied receptors as described under Experimental Procedures. Values are means ± standard error of the mean (n = 6–9).

DISCUSSION

The main findings of this study are as follows: (a) in studies of isolated enzyme, chlormadinone acetate prob-

ably competes for the ouabain binding site of Na^+,K^+-ATPase; and (b) chlormadinone acetate has limited solubility under the conditions used in muscle organ baths, and interpretation of data obtained under these conditions should be made with caution.

Many substances inhibit Na^+,K^+-ATPase activity and prevent or retard [^{3}H]ouabain binding to the enzyme. These two facts alone, however, are not sufficient evidence that the substance is binding directly to the ouabain binding site. It is quite likely that many of the substances are acting at an allosteric site or even nonspecifically. One cannot absolutely prove (short of isolation and comparison of peptides covalently attached to both the test compound and to ouabain) that a substance is acting at the same site as ouabain. However, we suggest that, in addition to the inhibition of Na^+,K^+-ATPase and [^{3}H]ouabain binding, the following minimal criteria should be fulfilled before such a hypothesis is made. (a) The substance should "chase" the radiolabel from the enzyme-[^{3}H]ouabain complex at the same rate as do ouabain and other cardiac glycosides. Satisfaction of this criterion does not prove that the substance binds at the same site as ouabain, but does indicate that the substance is not destroying the enzyme to cause ouabain release. For example, palmitylcarnitine inhibits both Na^+,K^+-ATPase and [^{3}H]ouabain binding in a dose-dependent manner. Palmitylcarnitine, however, greatly increases the rate of decomposition of the preformed enzyme-ouabain complex (11). Palmitylcarnitine does not bind to the ouabain site but acts nonspecifically to denature or solubilize the enzyme. (b) The substance should interact at a single kinetic class of sites on a purified enzyme, since this is what is found for cardiac glycosides and aglycones. If the substance is a reversible inhibitor of enzyme activity, this is easily determined by analysis of the shape of the dose-response curve or by a Scatchard-type transformation. (c) Since the affinity of cardiac glycosides and aglycones for Na^+,K^+-ATPase is regulated by physiological ligands, the binding and/or inhibition by the putative "ouabain-like" substance should be regulated in the same manner, which would indicate that the substance binds to the same form of the enzyme as ouabain. Specifically, the inhibition should be noncompetitive with respect to ATP and competitive with respect to potassium (up to 4–5 mM). In addition, the affinity of the enzyme for potassium derived from such a kinetic analysis should agree quantitatively with the affinity derived from studies of [^{3}H]ouabain binding or inhibition by aglycones. If a compound satisfies the criteria listed above, it is reasonable to presume that it binds to the same site on Na^+,K^+-ATPase as does ouabain. If not, the substance is probably acting at allosteric or nonspecific site(s). Since chlormadinone acetate satisfies these criteria it is probably binding to the same site on Na^+,K^+-ATPase as ouabain.

Chlormadinone acetate also resembles ouabain and ouabagenin in another respect. It is well known that Na^+,K^+-ATPase isolated from hearts of different species exhibits different sensitivities to cardiac glycosides (23). The Na^+,K^+-ATPase isolated from guinea pig or rabbit heart has a 10-fold lower affinity for cardiac glycosides than does enzyme isolated from dog or cat heart. The enzyme isolated from rat heart has a 100-fold lower affinity than the enzyme isolated from cat heart. Chlormadinone acetate possesses the same relative differences in sensitivity with respect to inhibition of Na^+,K^+-ATPase.

Na^+,K^+-ATPase is an unusual drug receptor, since its affinity for cardiac glycosides is regulated by allosteric sites. In the presence of magnesium plus ATP plus sodium, the enzyme has a high affinity for ouabain. In the presence of magnesium plus ATP plus potassium, the enzyme has a low affinity for ouabain. Sodium and potassium appear to compete for this allosteric site (14). One interpretation of these results is that occupation of the allosteric site by potassium causes a conformational change in the enzyme which occludes the ouabain binding site in some way, rendering it relatively inaccessible to the drug. If this is so, compounds that inhibit by binding to the ouabain binding site will display competitive inhibition with respect to potassium.

Analogues of cardiac glycosides have been used to determine which structural features are necessary for inhibition of enzyme activity. Thomas *et al.* (24) have suggested that all that might be needed is a —CH═CH—C═*A* attached to C-17 of the steroid nucleus. The symbol *A* represents either a carbonyl oxygen or a nitrile nitrogen. Fullerton *et al.* (25) in an elegant study have synthesized and determined the x-ray structure of a series of analogues of digitoxigenin. They found that the logarithm of the I_{50} of the compounds correlated very well with the position of the side-group carbonyl oxygen atom or nitrile nitrogen atom as measured from the carbonyl oxygen atom of digitoxigenin. Chlormadinone acetate is not a cardiac glycoside, yet it appears to bind to the cardiac glycoside receptor.

It is interesting in this respect to compare chlormadinone acetate with other compounds which have structures significantly different from that of cardiac glycosides but which inhibit [^{3}H]ouabain binding and enzyme activity. Sanguinarine, a benzophenathridine alkaloid, inhibits Na^+,K^+-ATPase. However, its inhibition has been reported to be uncompetitive with respect to sodium and potassium (26), and the enzyme from hearts of cats, guinea pigs, rabbits, and rats is equally sensitive (27). On the basis of these data, we suggest that sanguinarine is not binding to the ouabain site. On the other hand, cassaine, an erythrophleum alkaloid, shows the same relative species sensitivity as do chlormadinone acetate and cardiac glycosides. Inhibition by cassaine appeared to be noncompetitive with respect to potassium, but other experiments indicate that it is probably binding to the ouabain site (28). Prednisolone-3,20-bisguanylhydrazone, a reversible inhibitor of Na^+,K^+-ATPase, is competitive with respect to potassium (29). Binding studies, using radioactively labeled prednisolone-3,20-bisguanylhydrazone, have clearly shown that this compound is regulated in the same manner as ouabain (30). Furthermore, unlabeled ouabain chases labeled prednisolone-3,20-bisguanylhydrazone from its receptor, and unlabeled prednisolone-3, 20-bisguanylhydrazone chases [^{3}H]ouabain from its receptor. Unlike chlormadinone acetate and cassaine, its species sensitivity is different from that of cardiac glycosides. Na^+,K^+-ATPase isolated from guinea pig is sensitive, but the same enzyme isolated from dog, rat, and cat is relatively insensitive (31). There-

fore, although prednisolone-3,20-bisguanylhydrazone is probably binding to the ouabain site, the amino acid residues to which it binds are probably different from the amino acid residues to which ouabain binds.

On the basis of the foregoing there seems to be little doubt that chlormadinone acetate interacts with the ouabain binding sites of isolated Na^+,K^+-ATPase. However, there is an apparent discrepancy between the present observations that chlormadinone acetate failed to interfere with the binding of ouabain to its receptor in isolated muscles and failed to antagonize the inotropic action of ouabain with previous observations (8) that chlormadinone acetate, like ouabain, increased the internal sodium content of guinea pig atrium and rat diaphragm and affected sugar transport. The observations on the isolated heart are also apparently inconsistent with the existence of a common binding site for chlormadinone acetate and ouabain suggested by the present studies in isolated muscles. However, spectrophotometric measurements indicate that chlormadinone acetate concentrations of only 0.5–4 μM can be achieved in the tissue baths employed in the present experiments, levels that are insufficient to have much effect on ouabain binding sites, although such concentrations did affect Na^+ and K^+ levels in intact tissue (8). The reason why chlormadinone acetate is able to alter sodium and sugar transport in intact muscles and yet not affect ouabain binding or action of ouabain in the intact muscle strips is still not clear. However, the concentrations required to alter sodium and sugar transport in the tissue are significantly lower than those needed for inhibition of isolated Na^+,K^+-ATPase and suggest that measurement of the ion content of intact muscle may for some reason inadequately reflect at least quantitatively the activity of the sodium pump or the Na^+,K^+-ATPase in intact muscle.

ACKNOWLEDGMENTS

We extend our sincere appreciation to Drs. Frank S. LaBella and Ivan Bihler of the University of Manitoba for supplying chlormadinone acetate and for their review and critique of the manuscript. We should also like to thank Ms. Pam Smith for typing and editing.

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