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CORRELATION BETWEEN MICROSOMAL ($Na^+ + K^+$)-ATPase ACTIVITY AND [^{3}H]OUABAIN BINDING TO HEART TISSUE HOMOGENATES

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Summary

ATP plus Mg^{2+} plus Na^+ supported [^{3}H]ouabain binding to canine left ventricular tissue homogenates and microsomal ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity from the same tissue were measured. A linear relationship was found between the initial velocity of [^{3}H]ouabain binding to tissue homogenates and microsomal ($Na^+ + K^+$)-ATPase activity from the same tissue in the presence and absence of in vivo bound digoxin. In vivo bound digoxin reduced both measurements. With tissue from digoxin-free hearts, a linear relationship was also obtained between the initial velocity and the maximum level of [^{3}H]ouabain binding to tissue homogenate. Binding of [^{3}H]-ouabain to whole tissue homogenate is a convenient method for estimating ($Na^+ + K^+$)-ATPase activity in small left ventricular biopsy samples.

Introduction

($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is a membrane-bound enzyme involved in the coupled, active transport of Na^+ and K^+ and is specifically inhibited by cardiac glycosides [1,2]. There is considerable evidence that the positive inotropic action of cardiac glycosides is mediated through the inhibition of cardiac ($Na^+ + K^+$)-ATPase. Part of this evidence is that cardiac ($Na^+ + K^+$)-ATPase is partially inhibited during the positive inotropic effect of cardiac glycosides either in intact animals or in perfused heart preparations [3—6].

Cardiac (Na^+, K^+)-ATPase activity is usually measured in partially purified membrane fractions. Purification is necessary because the large amounts of myofibrillar ATPase present in the native tissue significantly mask ($Na^+ + K^+$)-ATPase activity. Thus in cardiac tissue, partial purification is necessary to remove much of interfering myofibrillar ATPase. Since purification is wasteful,

requiring gram quantities of tissue, only a single point determination of ($Na^+ + K^+$)-ATPase activity is possible for each heart, and control ($Na^+ + K^+$)-ATPase activity must be established in a separate control group. Furthermore, correspondence between tissue and the partially purified preparation is uncertain since during purification, important subfractions may be discarded and the drug receptor complex could dissociate. A method of estimating ($Na^+ + K^+$)-ATPase which needs only milligram amounts of tissue without purification would therefore be of advantage.

The work of Ku et al. [6] suggested that initial velocity of [^{3}H]ouabain binding to tissue homogenate might be a convenient method of estimating ($Na^+ + K^+$)-ATPase activity in the presence of large amounts of myofibrillar ATPase. The present study was therefore undertaken to investigate the relationship between the initial velocity of [^{3}H]ouabain binding to dog heart muscle homogenate and the ($Na^+ + K^+$)-ATPase activity of a partially purified microsomal preparation of the same tissue.

Materials and Methods

A biopsy drill [7] was used to obtain epicardial and mid-wall left ventricular tissue samples from anesthetized open-chested dogs. (Surgical instrumentation has been described previously [3]). Control biopsies were taken from β-blocked (2 mg/kg practolol) animals prior to commencement of [^{3}H]digoxin (specific activity 11.7 Ci/mol) infusion (1.88 μg/kg/min). The last biopsy was taken when digitalis toxicity developed (4 consecutive premature ventricular contractions). [^{3}H]Digoxin was used since we were interested in determining the relationship between the inotropic effects of digoxin, the subcellular distribution of digoxin, and ($Na^+ + K^+$)-ATPase activity, as measured by the [^{3}H]-ouabain binding method (manuscript in preparation). The animal was then sacrificed by removal of the heart. Sixteen animals were given digoxin prior to sacrifice, while two animals received only saline. All tissue taken was rinsed in chilled saline, blotted dry, then placed in dry ice (—70° C) until analysis.

[^{3}H]Ouabain binding

Left ventricular biopsy tissue (15—25 mg) was thawed, then homogenized in 1.15 ml of 0.32 M sucrose using a chilled, motor-driven Potter Elvehejm apparatus. Binding of [^{3}H]ouabain was determined by incubating the homogenate (0.5 ml) with 10^{-8} M [^{3}H]ouabain (specific activity 12.7 Ci/mol in the presence of 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 3 mM Tris-ATP, and 25 mM Tris · HCl buffer (pH 7.4) in a total volume of 5 ml at 37° C. The binding reaction was started by adding the homogenate to the pre-warmed incubation mixture. 0.5 ml aliquots were removed from the incubation mixture at 2.5, 5, 7.5, 10, 12.5, 15, and in some experiments at 120, 180 and 240 min. The aliquots were filtered through Millipore filters (0.45 μM) to separate unbound [^{3}H]ouabain from tissue-bound [^{3}H]ouabain which was trapped on the filter. The filters were washed twice with 2 ml of ice-cold Tris · HCl 25 mM buffer, pH 7.4, and dissolved in Bray's counting solution. The radioactivity retained on the filter was assayed using liquid scintillation counting and corrected for quenching by the automatic external standard channels ratio method. Non-specific [^{3}H]-

ouabain binding was determined in the absence of ATP and substracted from total binding to calculate specific ATP-supported binding. In this report, binding, unless otherwise stated, refers to specific, ATP-dependent component of [^{3}H]ouabain binding. [^{3}H]Ouabain binding was not corrected for [^{3}H]digoxin which could have been carried over into the incubation mixture with the heart tissue. Contribution of tissue-bound [^{3}H]digoxin to the radioactivity count would have been negligible, since its specific activity was very small (one thousandth) compared with that of [^{3}H]ouabain. This is confirmed by the low level of nonspecific binding to extent of 0.05 — 0.1 pmol/mg of protein.

Due to the small size of the biopsy samples, only single determinations of [^{3}H]ouabain binding were performed on samples of heart tissue obtained prior to sacrifice.

Preparation and measurement of ($Na^+ + K^+$)-ATPase

Methods previously described [3] were used to obtain and assay ($Na^+ + K^+$)-ATPase preparations from the left ventricular muscle of dogs sacrificed at toxicity. ATPase activities were measured by colorimetric determination of P_i release. Total ATPase activity was determined in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, 1 mM EGTA, 50 μg enzyme protein in 50 mM Tris · HCl buffer, pH 7.4, at 37° C. Mg^{2+}-ATPase activity was measured in the absence of Na^+ and K^+. ($Na^+ + K^+$)-ATPase activity was calculated by subtraction of Mg^{2+}-ATPase activity from the total, with correction for nonenzymatic P_i relase. Protein was measured by the method of Lowry et al. [14]. Each determination of ($Na^+ + K^+$)-ATPase activity was done in duplicate and the data point represents the average of two determinations which differed by less than 8%.

Results

Binding of [^{3}H]ouabain to tissue homogenate

[^{3}H]Ouabain binding to ($Na^+ + K^+$)-ATPase preparations has been shown to follow second order kinetics [8]. The amount of [^{3}H]ouabain maximally bound was linearly related to ($Na^+ + K^+$)-ATPase specific activity [9]. In the present experiments, the concentration of ouabain [10^{-8} M) was in excess, so that its free concentration changed by less than 10% during the course of incubation. Under these conditions, the kinetics of the binding reaction become pseudo-first-order, and the rate of formation of the ouabain-receptor complex is proportional to the concentration of free binding sites. Fig. 1 illustrates the time course of binding to homogenates of left ventricular tissue obtained from the same animal at various stages of digoxin infusion (see Methods). The initial velocity of ouabain-receptor complex formation was found to be constant and therefore the initial concentration of free binding sites could be estimated from the linear portion of the binding curve. Reproducibility in determining the initial velocity of binding was examined in five biopsies taken in rapid succession from a single heart. Coefficient of variation in the calculated initial velocity of [^{3}H]ouabain binding was 3%.

Decrease of free binding sites by digoxin is reflected by the diminished initial velocity of [^{3}H]ouabain binding to homogenates of tissue obtained during

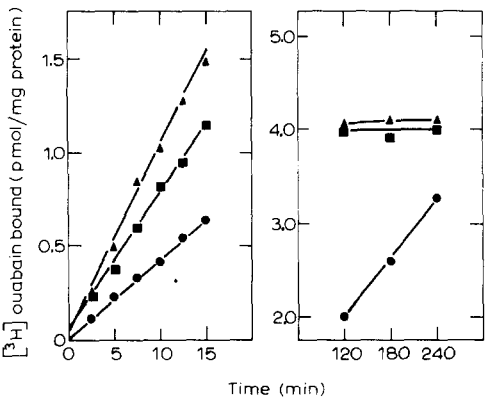

Fig. 1. Typical time course of [^{3}H]ouabain binding to canine left ventricular homogenate. All three biopsies were obtained from the same heart ▲, before digoxin was given: ■, during inotropic phase of digoxin (1.8 μg/kg/min) infusion and ●, at toxicity. Amount of [^{3}H]ouabain bound was measured as described under Methods and the values shown are corrected for non-specific binding.

digoxin infusion. As the incubation proceeds and more sites become occupied, the rate of appearance of [^{3}H]ouabain-receptor complex declines. In digoxin-free tissue and tissue where in vivo bound digoxin caused less than 25% reduction in the initial velocity of [^{3}H]ouabain binding, a maximal level of [^{3}H]ouabain-receptor was reached. This maximal binding reflects total amount of binding sites and would be expected to be the same for all three homogenates from the same heart (Fig. 1). Time taken to reach steady state (maximal binding), however, is longer for homogenates containing bound digoxin, and in homogenates where initial velocity of [^{3}H]ouabain binding was reduced by more than 25% by digoxin, the duration of incubation was not long enough for maximal ouabain binding to develop. The rate limiting process in reaching the steady state of binding is the dissociation of digoxin-receptor complex. Under conditions which favor binding, dissociation of digitalis-receptor complex is known to be slower than the association reaction [10,11].

Relationship between the initial velocity of [^{3}H]ouabain binding and ($Na^+ + K^+$)-ATPase specific activity

The ability to measure the initial velocity of [^{3}H]ouabain binding to tissue homogenate offers the possibility of investigating the relationship between this measurement and the ($Na^+ + K^+$)-ATPase activity of partially purified preparations of the same tissue.

Binding of [^{3}H]ouabain to canine heart tissue homogenate was measured as described under Methods. In every case, the plot of the amount of [^{3}H]ouabain bound against time was rectilinear during the first 15 min of incubation. Initial velocity of binding was calculated as the slope of the least squares regression line of [^{3}H]ouabain bound (pmol/mg protein) against time (min).

Fig. 2. shows that there is a strong linear relationship ($r = 0.89$, $P < 0.01$) between the initial velocity of [^{3}H]ouabain binding to tissue homogenate and microsomal ($Na^+ + K^+$)-ATPase activity, prepared by the method of Akera et al. [4], as modified by Goldman et al. [3].

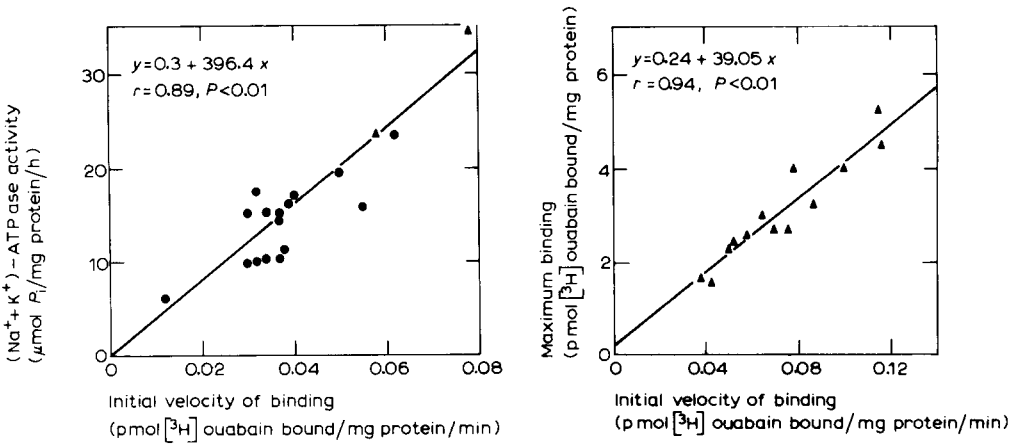

Fig. 2. Relationship between the initial velocity of [^{3}H]ouabain binding to dog left ventricular tissue homogenate and microsomal ($Na^+ + K^+$)-ATPase activity in the same tissue. Both measurements were made on heart tissue taken at the same time from dogs free of digoxin (▲) and from dogs given intravenous digoxin (1.8 μg/kg/min) (●). Amount of [^{3}H]ouabain bound to tissue homogenate was measured as described under Methods. Initial velocity of binding was taken as the slope of the least squares regression line of [^{3}H]ouabain bound (pmoles/mg protein) against time (min). ($Na^+ + K^+$)-ATPase activity is expressed in μmoles P_i/mg protein/h and was determined as described under Methods.

Fig. 3. Relationship between the initial velocity of [^{3}H]ouabain binding and the maximum level of [^{3}H]ouabain bound to the same homogenate of left ventricular tissue taken from digoxin-free hearts. Amount of [^{3}H]ouabain bound was measured as described under Methods. Initial velocity of binding was taken as the slope of the least squares regression line of [^{3}H]ouabain bound (pmol/mg protein) against time (min). Typical maximum level of [^{3}H]ouabain bound is illustrated by ▲ and ■ in the right panel of Fig. 1.

Relationship between the maximal level and the inital velocity of [^{3}H]ouabain binding

In tissue which has not been previously exposed to digitalis, two features of binding, namely the initial velocity and the maximal level, should both reflect the total numer of receptor sites. A linear correlation with high level of significance ($r = 0.94$, $P < 0.01$) was found between the initial velocity and the maximal level of [^{3}H]ouabain binding to tissue homogenate (Fig. 3).

Discussion

Binding of cardiac glycosides to membrane preparations containing ($Na^+ + K^+$)-ATPase has been studied extensively [8—13]. In such preparations ATP-dependent Mg^{2+} plus Na^+ supported [^{3}H]ouabain binding bears a linear relationship to ($Na^+ + K^+$)-ATPase activity in the presence or absence of other cardiac glycosides. The present study has extended this relationship by correlating [^{3}H]ouabain binding to whole tissue homogenates with ($Na^+ + K^+$)-ATPase activity of a partially purified microsomal fraction from the same tissue. Two features of [^{3}H]ouabain binding to tissue homogenate were found to reflect ($Na^+ + K^+$)-ATPase activity. The initial velocity of binding was found to be linearly related to the microsomal ($Na^+ + K^+$)-ATPase activity, and the maximum or equilibrium level of binding closely correlated with the initial velocity of binding. In the light of the demonstrated relationship between digitalis

binding and $(Na^+ + K^+)$-ATPase activity in purified membraned preparation [9, 13], the present study indicates that the measurement of these parameters in purified preparations provides an accurate estimate of whole tissue digitalis binding sites and $(Na^+ + K^+)$-ATPase content. Methods employed to partially purify $(Na^+ + K^+)$-ATPase give rise to uncertainty regarding translocation of digitalis during the procedure. Use of surfactants and salt solutions of considerable ionic strength could disrupt the digitalis-receptor complex. The strong correlation that was found between initial velocity of [^{3}H]ouabain binding to tissue homogenate and microsomal $(Na^+ + K^+)$-ATPase from tissues which were exposed to digoxin in vivo indicates that, in the species examined, the digitalis-receptor complex is stable during the purification procedures.

Estimation of $(Na^+ + K^+)$-ATPase activity by measuring digitalis binding to whole tissue homogenate offers several methodological advantages for exploring in vivo digitalis interactions with its receptors. The relatively mild conditions of homogenization compared to conventional $(Na^+ + K^+)$-ATPase purification methods may enable investigation of in vivo digitalis binding to cardiac $(Na^+ + K^+)$-ATPase in species which form a less stable drug-receptor complex. In addition, since binding can be measured in small biopsy samples of heart tissue, $(Na^+ + K^+)$-ATPase activity can be estimated at various levels of pharmacological effect of digitalis in a single animal, and each animal can act as its own control. Furthermore, both free and total binding sites, corresponding to the residual and the total $(Na^+ + K^+)$-ATPase activity, in the presence of cardiac glycosides, may be determined on a single tissue sample.

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References

1 Schatzmann, H.J. (1953) Helv. Physiol. Pharmacol. Acta, 11, 346–354
2 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3–134
3 Goldman, R.H., Coltart, J.D., Friedman, J.P., Nola, G.T., Berke, D.K., Schweizer, E. and Harrison, D.C. (1973) Circulation 48, 830–838
4 Akera, T., Larsen, F.S. and Brody, T.M. (1970) J. Pharmacol. Exp. Ther. 173. 17–26
5 Besch, Jr., H.R., Allen, J.C., Glick, G. and Schwartz, A. (1970) J. Pharmacol. Exp. Ther. 174, 323–329
6 Ku, D., Akera, T., Pew, C.L. and Brody, T.M. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. 5, 185–200
7 Schwartz, K., Rey, P., Bui-Mong-Hung and De Mendonca, M. (1973) J. Mol. Cell Cardiol. 5, 235–246
8 Erdman, E. and Schoner, W. (1973) Biochim. Biophys. Acta 307, 386–398
9 Hansen, O. (1971) Biochim. Biophys. Acta, 233, 122–132
10 Lindemayer, G.E. and Schwartz, A. (1973) J. Biol. Chem., 248, 1291–1300
11 Lane, L.K., Copenhaver, J.H., Jr., Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 7197–7200
12 Schwartz, A., Matsui, H. and Laughter, A.H. (1968) Science, 159, 323–325
13 Allen, J.C., Martinez-Maldonado, M., Eknoyan, G., Sulhi, W.N. and Schwartz, A. (1971) Biochem. Pharmacol., 20, 73–80
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265–275