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Erythrosin B inhibits high affinity ouabain binding in guinea-pig heart Na^+-K^+-ATPase without influence on cardiac glyoside induced contractility

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1 Binding of [^{3}H]-ouabain to guinea-pig heart membranes enriched in Na^+-K^+-ATPase revealed two different cardiac glycoside binding sites. High affinity binding was obtained at a $K_D = 2.2 \times 10^{-7}$ mol l^{-1} (B_{max} = 16.8 pmol ouabain mg^{-1} protein) whereas low affinity ouabain binding occurred at a $K_D > 10^{-6}$ mol l^{-1}.

2 To discover whether the two ouabain binding sites are functional in guinea-pig heart muscle, erythrosin B, an inhibitor of the high affinity ouabain binding in rat brain tissue, was tested in guinea-pig isolated heart muscle preparations. Erythrosin B proved to be a potent inhibitor of the Mg^{2+}(Na^+)-dependent-, as well as Na^+-K^+-activated ATPase ($ID_{50} = 9 \times 10^{-6}$ mol l^{-1}). Contractility of guinea-pig isolated papillary muscles, however, was not influenced by erythrosin B in concentrations up to 1×10^{-5} mol l^{-1}. Only very high concentrations (4×10^{-4} mol l^{-1}) resulted in a slightly negative inotropic effect (about 20%).

3 Erythrosin B dose-dependently inhibited [^{3}H]-ouabain binding to the Na^+-K^+-ATPase ($K_D = 3.6 \times 10^{-6}$ mol l^{-1}). In a concentration of 1×10^{-5} mol l^{-1} the dye abolished high affinity [^{3}H]-ouabain binding without affecting the low affinity binding sites.

4 In contrast, in guinea-pig isolated atria, no functional antagonism between erythrosin B (5×10^{-5} mol l^{-1}) and ouabain was observed.

5 As there is a coincidence between the high affinity binding ($K_D = 2.2 \times 10^{-7}$ mol l^{-1}) and the concentration for half maximum inotropic effects of ouabain ($ED_{50} = 1.6 \times 10^{-7}$ mol l^{-1}), the lack of effect of erythrosin B on ouabain-induced inotropy may be caused by an inaccessibility of the dye to the (internal) ATP-site of the Na^+-K^+-ATPase.

Introduction

The Na^+-K^+-ATPase is generally accepted as the most specific binding site ('receptor') for cardiac glycosides in the heart. However, the coupling of this interaction with the inotropic effect is still the subject of discussion. A highly favoured theory is the causal relationship between the binding to and inhibition of the Na^+-K^+-ATPase by cardiac glycosides. This results in an increase in the intracellular Na^+-concentration which, via the Na^+/Ca^{2+}-exchange, is linked to an increase in the intracellular Ca^{2+} concentration, thereby accounting for the positive inotropic effect of these drugs (for references see Schwartz *et al.*, 1975; Akera & Brody, 1977). However, there is some evidence, that cardiac glycosides in low but positive inotropic concentrations do not inhibit Na^+,K^+-transport in intact heart muscle preparations (Cohen *et al.*, 1976; Noack *et al.*, 1979; Godfraind, 1981; Brown & Erdmann, 1982, Fricke *et al.*, 1982).

Recent results indicate that, under different experimental conditions in different species and tissues, at least two cardiac glycoside binding sites may exist (Inagaki *et al.*, 1974; Fricke & Klaus, 1977; 1978; Erdmann *et al.*, 1980; Silbergeld, 1981; Erdmann, 1983; Lazdunski *et al.*, 1983). It has been suggested that the high affinity, low capacity binding of cardiac glycosides may be related to the positive inotropic action of these drugs, whereas the low affinity, high capacity binding of cardiac glycosides is connected to

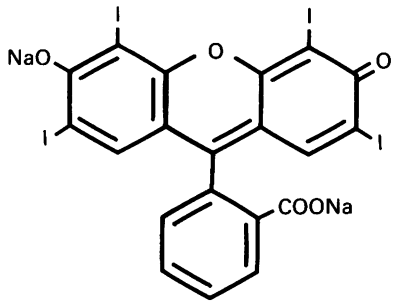

Figure 1 Structure of erythrosin B.

the inhibition of the Na^+-K^+-ATPase and hence to an impairment of the Na^+,K^+-homeostasis resulting in arrhythmias and contracture (Erdmann *et al.*, 1980; Erdmann, 1983; Werdan *et al.*, 1983).

Reports that erythrosin B (Figure 1), an artificial food dye, can specifically inhibit the high affinity ouabain binding in rat brain tissue (Silbergeld, 1981; Swann, 1982; Hnatowich & La Bella, 1982), indicated that this drug might be a useful tool to unravel the functional significance of the two ouabain binding sites found in isolated heart muscle preparations. For this reason, the effects of erythrosin B and its interaction with ouabain was studied in guinea-pig heart muscle preparations and Na^+-K^+-ATPase.

Methods

Preparation of Mg^{2+}-dependent, Na^+-K^+-activated ATPase

Microsomes enriched in cardiac Mg^{2+},Na^+-K^+-ATPase from guinea-pigs of either sex (200–300 g) were freshly prepared before use as previously described (Fricke & Klaus, 1974). The final enzyme preparation, stored in an imidazole-HCl buffer (100 mmol l^{-1} imidazole HCl, 1 mmol l^{-1} Na_2-EDTA, pH 7.4), was used for the experiments.

The Na^+-K^+-ATPase activity was in the range of 5–8 μmol ATP hydrolyzed per h and mg protein at 37°C. About 80–90% of the total activity was inhibited by 1×10^{-3} mol l^{-1} ouabain.

ATPase activity assay

Mg^{2+}-dependent and Na^+-K^+-stimulated ATPase activities were determined at 37°C in the presence of increasing concentrations of erythrosin B. Usually 10 μg of protein was preincubated for 10 min in 100 mmol l^{-1} imidazole HCl buffer, pH 7.4, containing: (mmol l^{-1}) $MgCl_2$ 5, NaCl 100, KCl 5 and Na_2-EDTA 1. The reaction was started by the addition of ATP, the final concentration being 2 mmol l^{-1}. An identical assay was performed in the absence of KCl (Mg^{2+}(Na^+)-dependent ATPase activity). After 30 min, inorganic phosphate was determined by the method of Eibl & Lands (1969). Each assay was performed in duplicate. Na^+-K^+-ATPase activity was calculated as the difference between total and Mg^{2+} (Na^+)-dependent activity.

[^{3}H]-ouabain binding

The binding studies were carried out as described by Erdmann & Schoner (1973, 1974). Briefly, the experiments were performed by incubation of the Na^+-K^+-ATPase (0.15 mg protein) in the presence of 5 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} imidazole/PO_4^{3-}, 5.5 nmol l^{-1} [^{3}H]-ouabain and increasing amounts of unlabelled ouabain (5–4000 nmol l^{-1}) in 100 mmol l^{-1} imidazole HCl buffer, pH 7.4 (total volume = 2 ml). After 30 min of incubation (steady state condition), bound ouabain was separated from free drug by a rapid filtration technique (Whatman GF/C glass filter membranes). After washing the filter twice with 10 ml of ice-cold distilled water the filters were added to a scintillation mixture (0.3% PPO, 25.7% Triton X-100, 74% xylene) and counted for radioactivity in an Intertechnique SL 30 liquid scintillation spectrometer. Non-specific binding was determined in the presence of high concentrations of unlabelled ouabain (1×10^{-3} mol l^{-1}) and was found to be less than 1% of total radioactivity. All experiments were performed in duplicate assays.

Measurement of inotropic action

Inotropic action was evaluated on electrically stimulated right papillary muscles (60 min^{-1}, 3 ms, 50 V) or left atria (120 min^{-1}, 6 ms, 2 × threshold voltage), isolated from guinea-pig hearts (guinea-pigs of either sex, 200–250 g). Contractile force was measured isometrically by means of a force-displacement-transducer and recorded after amplification (Fleck, Mainz, F.R.G.) on a thermorecorder (Hellige Servomed, Freiburg, F.R.G.). The atria (resting tension: 1.0 g = 9.8 mN) or papillary muscles (resting tension: 0.5 g = 4.9 mN) were allowed to stabilize at 30°C for about 60 min in an organ bath containing 10 ml of a modified Tyrode solution (mmol l^{-1}): Na^+ 149, K^+ 5.4, Ca^{2+} 0.9, Mg^{2+} 1.05, Cl^- 144, HCO_3^- 11.9, $H_2PO_4^-$ 0.42 and glucose 10.0, which was equilibrated with 95% O_2, 5% CO_2 to give a pH of 7.4.

Erythrosin B and ouabain were dissolved in distilled water and usually added in a cumulative manner at 30 min intervals until toxic effects (contracture, arrhythmias) were observed. The time intervals were chosen such as to allow a steady state of drug action.

Miscellaneous

Protein concentrations were determined by the method of Lowry *et al.* (1951) using Labtrol (Merz & Dade, München, F.R.G.) as a standard.

K_D values for direct binding studies with [^{3}H]-ouabain were calculated according to Wellstein & Palm (1984) using a nonlinear least square fitting procedure or to Noel & Godfraind (1984), using a derivative-free nonlinear regression programme (PAR, BMDP Statistical software, Inc., U.S.A.). Furthermore, Scatchard-plots were used for purposes of comparison (Scatchard, 1949). Indirect K_D values were calculated according to Erdmann & Schoner (1974). The concentrations for half maximum inotropic response (ED_{50}) and half maximum inhibition of the Na^+-K^+-ATPase (ID_{50}) were calculated from the individual concentration-effect-curves as proposed by Hafner *et al.* (1977). All data were analysed by standard statistical methods (mean value and s.e.mean, regression analysis).

Materials

[^{3}H]-ouabain, sp. act. 666 GBq mmol^{-1} was purchased from New England Nuclear (Dreieich, F.R.G.). Erythrosin B and Na_2-ATP were obtained from Serva (Heidelberg, F.R.G.). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.).

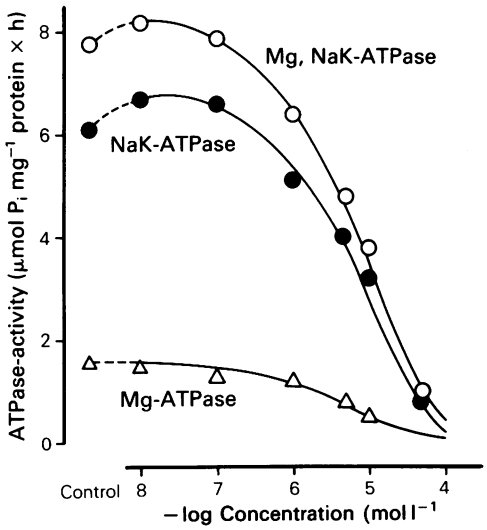

Figure 2 Effects of increasing concentrations of erythrosin B on the Mg^{2+}(Na^+)-dependent- (△), Mg^{2+},Na^+-K^+-dependent- (○) and on the Na^+-K^+-dependent ATPase activity (●) from guinea-pig heart. Enzyme activities were assayed for 30 min at 37°C in the presence of $MgCl_2$ 5 mmol l^{-1}, NaCl 100 mmol l^{-1} and ATP 2 mmol l^{-1} (Mg^{2+}(Na^+)-ATPase) or $MgCl_2$ 5 mmol l^{-1}, NaCl 100 mmol l^{-1}, KCl 5 mmol l^{-1} and ATP 2 mmol l^{-1} (Mg^{2+}, Na^+-K^+-ATPase) and are expressed in µmol P_i mg^{-1} protein × h. The means of 3 different experiments are given.

Results

Erythrosin B

Inotropic action In guinea-pig isolated papillary muscles erythrosin B in a concentration range of 5×10^{-7} mol l^{-1} to 1×10^{-5} mol l^{-1} did not significantly alter contractile force. Higher concentrations resulted in a slightly negative inotropic effect (up to about 20%) at the highest concentration tested (4×10^{-4} mol l^{-1}).

Mg^{2+}-dependent, Na^+-K^+-ATPase The effects of erythrosin B on myocardial Mg^{2+}-dependent, Na^+-K^+-activated ATPase were tested in a concentration range of 10^{-8} mol l^{-1} to 5×10^{-5} mol l^{-1}. Erythrosin B dose-dependently inhibited the Na^+-K^+-ATPase activity as well as the Mg^{2+}-dependent enzyme activity (Figure 2). From these experiments in either case a concentration for half maximum inhibition (ID_{50}) of 9×10^{-6} mol l^{-1} was calculated.

Binding to Na^+-K^+-ATPase On the assumption that unlabelled compounds bind to the same binding sites as their labelled analogues, the inhibition of [^{3}H]-ouabain binding to the Na^+-K^+-ATPase by un-

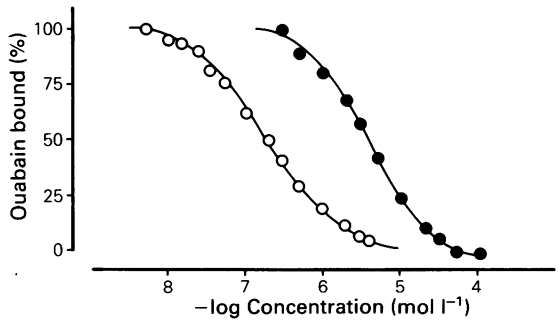

Figure 3 Inhibition of [^{3}H]-ouabain binding to guinea-pig heart Na^+-K^+-ATPase by unlabelled ouabain (○) and erythrosin B (●). Cardiac cell membranes (0.15 mg protein) incubated for 30 min at 37°C in 100 mmol l^{-1} imidazole HCl pH 7.4, $MgCl_2$ 5 mmol l^{-1}, imidazole/PO_4^{3-} 3 mmol l^{-1}, [^{3}H]-ouabain 5.5 nmol l^{-1} and increasing concentrations of the drugs indicated. Non-specific [^{3}H]-ouabain binding in the presence of 10^{-3} mol l^{-1} unlabelled ouabain was subtracted. Dissociation constants (K_D) calculated according to Erdmann & Schoner (1974): ouabain $K_D = 1.9 \times 10^{-7}$ mol l^{-1}; erythrosin B $K_D = 3.6 \times 10^{-6}$ mol l^{-1}. Means of 6 different assays are given.

labelled ouabain or other unlabelled compounds can be used to calculate the concentration for half maximum binding (K_D) (Erdmann & Schoner, 1974).

The K_D value for ouabain (high affinity binding) obtained in this way was 1.9×10^{-7} mol l^{-1} and corresponds to the K_D value of 2.0×10^{-7} mol l^{-1}, which was obtained directly by the analysis according to Scatchard (1949) from the same experiments. Erythrosin B in a concentration range of 5×10^{-7} mol l^{-1} to 1×10^{-4} mol l^{-1} dose-dependently inhibited [^{3}H]-ouabain binding. From these competition-curves an indirect K_D value for erythrosin B of 3.6×10^{-6} mol l^{-1} was obtained (Figure 3).

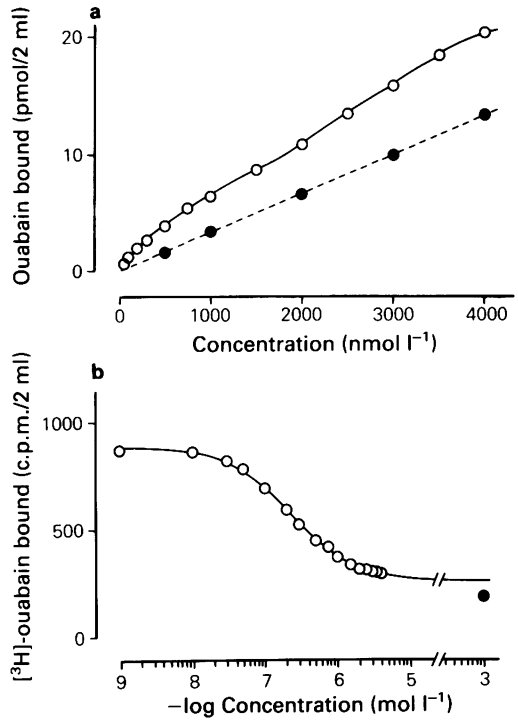

Figure 4 Binding of [^{3}H]-ouabain to guinea-pig heart Na^+-K^+-ATPase. Cardiac cell membranes (0.15 mg protein) incubated for 30 min at 37°C in 100 mmol l^{-1} imidazole HCl pH 7.4, 5 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} imidazole/PO_4^{3-}, 5.5 nmol l^{-1} [^{3}H]-ouabain, and increasing concentrations of unlabelled ouabain (5×10^{-9}–10^{-3} mol l^{-1}, [^{3}H]-ouabain binding in the presence of 10^{-3} mol l^{-1} unlabelled ouabain considered to be non-specific). Further conditions as described under Methods. (a) Saturation binding of ouabain; (b) Binding of [^{3}H]-ouabain as a competition isotherm, demonstrating two different populations of ouabain binding sites (as calculated by the method of Wellstein & Palm, 1984). A high affinity binding site (B_{max} = 16.8 pmol mg^{-1} protein, $K_D = 2.2 \times 10^{-7}$ mol l^{-1}) and a low affinity binding site indicating a K_D value of $> 10^{-6}$ mol l^{-1}. Open symbols represent total binding, closed symbols the non-specific binding of ouabain. The means of 6 different assays are given.

Erythrosin B – ouabain interactions

Binding to Na^+-K^+-ATPase Incubation of guinea-pig heart microsomes with 5.5 nmol l^{-1} [^{3}H]-ouabain and increasing concentrations of unlabelled ouabain (5×10^{-9} mol l^{-1} to 1×10^{-3} mol l^{-1}) resulted in a saturable cardiac glycoside binding, which is not monotonic over the whole ouabain concentration range studied. These data analysed according to the method described by Wellstein & Palm (1984) were compatible with the existence of two specific ouabain binding sites (Figure 4): one binding site, evident at low ouabain concentrations, had a K_D value of 2.2×10^{-7} mol l^{-1} and a maximum binding capacity (B_{max}) of 16.8 pmol ouabain mg^{-1} protein ('high affinity binding site'). The other binding site representing about 10% of the [^{3}H]-ouabain bound was obtained only at rather high ouabain concentrations. The K_D value which cannot be quantified exactly, was above 1×10^{-6} mol l^{-1} ('low affinity binding site'). Similar results were obtained when the data were analysed according to the method described by Noel & Godfraind (1984). Two populations of binding sites are also apparent in a Scatchard plot (1949). However, as this transformation can give erroneous interpretations (Munson & Rodbard,1983; Klotz, 1983), nonlinear analysis of the untransformed data is preferred (Wellstein & Palm, 1984).

Erythrosin B at concentrations that inhibited [^{3}H]-ouabain binding by about 50–80%, only inhibited the high affinity, low capacity cardiac glycoside binding site, and did not affect the low affinity component (Figure 5). Nonlinear analysis of the untransformed data according to Wellstein & Palm (1984) resulted in a K_D value of the high affinity ouabain binding in the *absence* of erythrosin B of 1.4×10^{-7} mol l^{-1}, (B_{max} = 7.3 pmol mg^{-1} protein). Erythrosin B in a concentration of 5×10^{-6} mol l^{-1} shifted this K_D value to 3.9×10^{-7} mol l^{-1} and in a concentration of 1×10^{-5} mol l^{-1} almost abolished high affinity ouabain binding ($K_D = 8.0 \times 10^{-7}$ mol l^{-1}). The maximum binding capacities under these conditions varied between 8.9 pmol mg^{-1} protein (5×10^{-6} mol l^{-1} erythrosin B) and 10.9 pmol mg^{-1} protein (1×10^{-5} mol l^{-1} erythrosin B). Analysis of the same data according to the proposal of Noel & Godfraind (1984), yielded similar results, indicating erythrosin B to be a specific inhibitor of the high affinity ouabain binding in guinea-pig heart.

Inotropic action The interaction of erythrosin B with ouabain was studied in guinea-pig isolated left atria. In the absence of erythrosin B ouabain dose-dependently increased contractility in this preparation in a concentration range of 5×10^{-8} mol l^{-1} to 4×10^{-7} mol l^{-1}

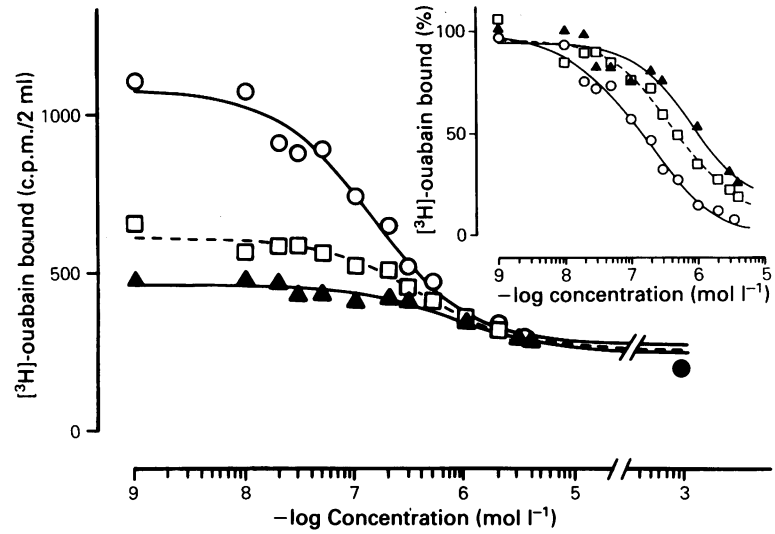

Figure 5 [^{3}H]-ouabain binding to guinea-pig heart Na^+-K^+-ATPase in the absence (○) and the presence of 5×10^{-6} mol l^{-1} (□) or 1×10^{-5} mol l^{-1} (▲) erythrosin B. Cardiac cell membranes (0.15 mg protein) incubated for 30 min at 37°C in 100 mmol l^{-1} imidazole HCl pH 7.4, $MgCl_2$ 5 mmol l^{-1}, imidazole/PO_4^{3-} 3 mmol l^{-1}, [^{3}H]-ouabain 5.5 nmol l^{-1}, and increasing concentrations of unlabelled ouabain (5×10^{-9}–10^{-3} mol l^{-1}). [^{3}H]-ouabain binding in the presence of 10^{-3} mol l^{-1} unlabelled ouabain (●) considered to be non-specific. Plotting the data as a competition isotherm according to the method of Wellstein & Palm (1984), the inhibition of the high affinity ouabain binding by increasing concentrations of erythrosin B is evident (see also insert). So calculated K_D value of ouabain in the absence of erythrosin B was $K_D = 1.4 \times 10^{-7}$ mol l^{-1} ($B_{max} = 7.3$ pmol mg^{-1} protein). Erythrosin B in concentrations of 5×10^{-6} mol l^{-1} and 1×10^{-5} mol l^{-1} shifted this value to $K_D = 3.9 \times 10^{-7}$ mol l^{-1} ($B_{max} = 8.9$ pmol mg^{-1} protein) and $K_D = 8.0 \times 10^{-7}$ mol l^{-1} ($B_{max} = 10.9$ pmol mg^{-1} protein), respectively. The low affinity ouabain binding seemed to be unaffected. Means of 3–4 different experiments are given.

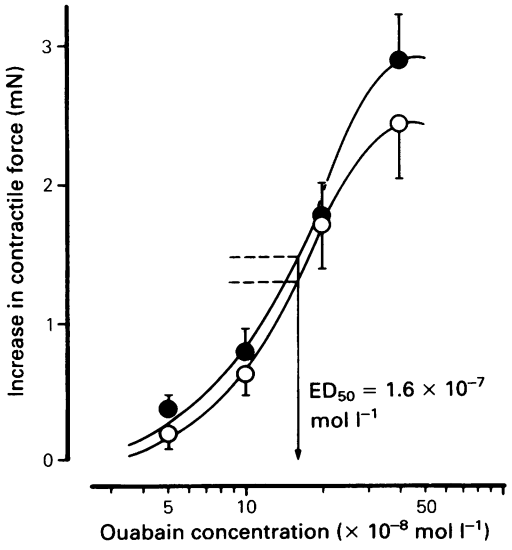

Figure 6 Increase in contractile force (ordinate scale, in mN) by ouabain (abscissa scale, in mol l^{-1}) in the presence (●) and absence (○) of 5×10^{-5} mol l^{-1} erythrosin B in guinea-pig isolated left atria. In either case a concentration of ouabain for half maximum response of 1.6×10^{-7} mol l^{-1} could be calculated. Means of 4 different experiments, each, are given; s.e.means shown by vertical lines.

with a maximum inotropic effect obtained of 2.5 ± 0.5 mN ($n = 4$).

In a second series of experiments ouabain was added to the atria after preincubation with erythrosin B, 5×10^{-5} mol l^{-1}, which at this concentration exerted no significant change in contractile force. Erythrosin B pretreatment did not modify ouabain-induced contractility (maximum effect = 2.9 ± 0.3 mN, $n = 4$). nor did it alter the effective concentration range of the cardiac glycoside (Figure 6).

Discussion

Although most studies on the binding of cardiac glycosides result in a saturable binding which involves one single class of binding sites (for references see Wallick *et al.*, 1979; Erdmann, 1981), recent reports on different species and tissues have focussed on the possible existence of at least two populations of cardiac glycoside binding sites (e.g. Inagaki *et al.*, 1974; Fricke & Klaus, 1977; Heller & Beck, 1978; Sweadner, 1979; Erdmann *et al.*, 1980; Erdmann, 1983; Lazdunski *et al.*, 1983). In heart muscle, the functional significance of these two binding sites has been related to the positive inotropic action of cardiac glycosides ('high affinity, low capacity binding') and

to the toxic action of these drugs ('low affinity, high capacity binding'), respectively (Erdmann, 1983; Werdan *et al.*, 1983). In contrast Lazdunski *et al.* (1983), who in cultured chick heart cells could also demonstrate two ouabain binding sites, suggested that the low affinity ouabain binding, which was related to the inhibition of the cardiac Na^+-K^+-ATPase, was probably responsible for both the cardiotonic and cardiotoxic effects of this drug. Finally, an intermediate interpretation is given by Adams *et al.* (1982) and Finet *et al.* (1983), who postulated from experiments on rat isolated heart muscle preparations, that the high affinity *and* the low affinity ouabain binding sites were involved in the inotropic action of this drug: low cardiac glycoside concentrations could cause an increased mobilization of intracellular calcium and higher ouabain concentrations result in an inhibition of the Na^+-K^+-pump and consequent activation of the Na^+-Ca^{2+}-exchange mechanism.

These controversial viewpoints of the mechanism of cardiac glycoside-induced positive inotropy may only be evaluated if specific inhibitors of either of the two ouabain binding sites can be established in heart muscle preparations. Recent reports on the partial antagonism of [^{3}H]-ouabain binding by erythrosin B in rat brain tissue (Silbergeld, 1981; Swann, 1982; Hnatowich & LaBella, 1982) offered such an opportunity. The present study proves erythrosin B to be an inhibitor of the Mg^{2+}-dependent as well as the Na^+-K^+-activated ATPase isolated from guinea-pig heart. The concentration for half maximum inhibition of the enzyme (ID_{50}) is somewhat higher than that obtained with ouabain in the same preparation ($ID_{50} = 3 \times 10^{-6}$ mol l^{-1}; Fricke, 1978). However, in guinea-pig isolated papillary muscles, erythrosin B, in contrast to ouabain, did not influence contractile force. Very high erythrosin B concentrations (about 1×10^{-4} mol l^{-1}) resulted in a decrease of contractile force.

On the basis of these results the interaction of erythrosin B with ouabain was studied on Na^+-K^+-ATPase-enriched membrane preparations and on left atria isolated from guinea-pig hearts. As has been demonstrated recently in different tissues and species (Heller & Beck, 1978; Sweadner, 1979; Erdmann *et al.*, 1980; Silbergeld, 1981; Hnatowich & LaBella, 1982; Erdmann, 1983; Lazdunski *et al.*, 1983) ouabain binding resulted in two different binding sites with the K_D value of the low affinity, high capacity ouabain binding being over 10 times higher than the K_D value of the high affinity, low capacity binding site. The effect of increasing concentrations of erythrosin B (up to 1×10^{-5} mol l^{-}) on the cardiac glycoside binding to myocardial Na^+-K^+-ATPase was to inhibit progressively the high affinity ouabain binding without affecting the low affinity ouabain binding site. These results confirm those of Silbergeld (1981) and Hnatowich & LaBella (1982) in rat brain membranes. However, in guinea-pig isolated atria a functional antagonism between ouabain and erythrosin B could not be found at concentrations that selectively inhibited the high affinity ouabain binding to cardiac Na^+-K^+-ATPase in the above studies.

If it were assumed that the high affinity ouabain binding in guinea-pig heart muscle is responsible for the inotropic action of this drug and that the low affinity binding corresponds to the inhibition of the Na^+-K^+-ATPase, the lack of effect of erythrosin B on whole tissue functions might appear to indicate that the high affinity binding is not involved in ouabain-induced inotropy and the low affinity cardiac glycoside binding is responsible for both the cardiotonic and cardiotoxic action of ouabain. This interpretation corresponds to the conclusion drawn by Lazdunski *et al.* (1983). However, the coincidence of the high affinity binding ($K_D = 2.2 \times 10^{-7}$ mol l^{-1}, cf. Figure 4) with the ouabain concentration for half maximum inotropic effects ($ED_{50} = 1.6 \times 10^{-7}$ mol l^{-1}, cf. Figure 6) argues against that interpretation. An alternative proposal is that the site of action of erythrosin B is intracellular. In the beating heart preparation, this is not accessible to the dye because of its limited permeability, whereas in broken cell preparations there is no such permeability barrier. An interaction of erythrosin B with the (internal) ATP-site of the Na^+-K^+-ATPase has already been postulated by Swann (1982).

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References

ADAMS, R.J., SCHWARTZ, A., GRUPP, G., GRUPP, I., LEE, S.W., WALLICK, E.T., POWELL, T., TWIST, V.W. & GATHIRAM, P. (1982). High affinity ouabain binding site and low-dose positive inotropic effect in rat myocardium. *Nature*, **296**, 167–169.

AKERA, T. & BRODY, T.M. (1977). The role of Na^+,K^+-ATPase in the inotropic action of digitalis. *Pharmac. Rev.*, **29**, 187–220.

BROWN, L. & ERDMANN, E. (1982). ^{3}H-ouabain binding and effects on force of contraction and ^{86}Rb-uptake at different stimulation frequencies in guinea-pig left atria. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **319** (Suppl), R42.

BROWN, L., ERDMANN, E. & THOMAS, R. (1983). Digitalis structure-activity relationship analyses. Conclusions from indirect binding studies with cardiac ($Na^+ + K^+$)-ATPase. *Biochem. Pharmac.*, **32**, 2767–2774.

COHEN, I., DAUT, J. & NOBLE, D. (1976). An analysis of the action of low concentrations of ouabain on membrane currents in Purkinje fibres. *J. Physiol.*, **260**, 75–103.

EIBL, H.J. & LANDS, W.C. (1969). A new sensitive determination of phosphate. *Anal. Biochem.*, **30**, 51–57.

ERDMANN, E. (1981). Influence of cardiac glycosides on their receptor. In *Handbook of Experimental Pharmacology*, Vol 56/I, Cardiac Glycosides. ed. Greef, K. pp. 337–380. Berlin/Heidelberg/New York: Springer.

ERDMANN, E. (1983). Evidence for two kinetically and functionally different types of cardiac glycoside receptors in the heart. *J. mol. cell. Cardiol.*, **15** (Suppl. 2), 46.

ERDMANN, E., PHILIPP, G. & SCHOLZ, H. (1980). Cardiac glycoside receptor, ($Na^+ + K^+$)-ATPase activity and force of contraction in rat heart. *Biochem. Pharmac.*, **29**, 3219–3229.

ERDMANN, E. & SCHONER, W. (1973). Ouabain-receptor interactions in ($Na^+ + K^+$)-ATPase preparations from different tissues and species. Determination of kinetic constants and dissociation constants. *Biochim. biophys. Acta*, **307**, 386–398,

ERDMANN, E. & SCHONER, W. (1974). Ouabain-receptor interactions in ($Na^+ + K^+$)-ATPase preparations. IV. The molecular structure of different cardioactive steroids and other substances and their affinity to the glycoside receptor. *Naunyn-Schmiedebergs Arch. Pharmac.*, **283**, 335–356.

FINET, M., GODFRAIND, T. & NOEL, F. (1983). The inotropic effect of ouabain and its antagonism by dihydroouabain in rat isolated atria and ventricles in relation to specific binding sites. *Br. J. Pharmac.*, **80**, 751–759.

FRICKE, U. (1978). Myocardial activity of inhibitors of the Na^+-K^+-ATPase: Differences in the mode of action and subcellular distribution pattern of N-ethylmaleimide and ouabain. *Naunyn-Schmiedebergs Arch. Pharmac.*, **303**, 197–204.

FRICKE, U., HOTTA, Y. & KLAUS, W. (1982). Reversible inhibition of $^{86}Rb^+(K^+)$-uptake in guinea-pig isolated cardiac muscle by digitoxigenin-3-tosyloxy-acetate, an active site-directed label of the Na^+-K^+-ATPase. *Naunyn-Schmiedebergs Arch. Pharmac.*, **321** (Suppl), R39.

FRICKE, U. & KLAUS, W. (1974). A simple preparation technique for a microsomal $Na^+ - K^+$-activated ATPase from cardiac tissues of different species. *Prep. Biochem.*, **4**, 13–29.

FRICKE, U. & KLAUS, W. (1977). Evidence for two different Na^+-dependent [^{3}H]-ouabain binding sites of a Na^+-K^+-ATPase of guinea-pig hearts. *Br. J. Pharmac.*, **61**, 423–428.

FRICKE, U. & KLAUS, W. (1978). Sodium-dependent cardiac glycoside binding: Experimental evidence and hypothesis. *Br. J. Pharmac.*, **62**, 255–257.

GODFRAIND, T. (1981). Stimulation and inhibition of the Na^+,K^+-pump by cardiac glycosides. In: *Handbook of Experimental Pharmacology*, Vol 56/I, *Cardiac Glycosides*. ed. Greeff, K. pp. 381–393. Berlin/Heidelberg/New York: Springer.

HAFNER, D., HEINEN, E. & NOACK, E. (1977). Mathematical analysis of concentration-response-relationships. *Arzneim.-Forsch.*, **27**, 1871–1873.

HELLER, M. & BECK, S. (1978). Interactions of cardiac glycosides with cells and membranes. Properties and structural aspects of two receptor sites for ouabain in erythrocytes. *Biochim. biophys. Acta*, **514**, 332–347.

HNATOWICH, M. & LABELLA, F.S. (1982). Light-enhanced inhibition of ouabain binding to digitalis receptor in rat brain and guinea-pig heart by the food dye erythrosine. *Mol. Pharmac.*, **22**, 687–692.

INAGAKI, C., LINDENMAYER, G.E. & SCHWARTZ, A. (1974). Effects of sodium and potassium on binding of ouabain to the transport adenosine triphosphatase. *J. biol. Chem.*, **249**, 5135–5140.

KLOTZ, I.M. (1983). Reply to: Number of receptor sites from Scatchard and Klotz graphs (Munson, P.J., Rodbard, D.). *Science*, **220**, 981.

LAZDUNSKI, M., KAZAZOGLOU, T., PONZIO, G., RENAUD, J.F. & ROSSI, B. (1983). The digitalis receptor in cardiac cells and its relation with positive inotropy. *J. mol. cell. Cardiol.*, **15** (Suppl 2), 46.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.I. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MUNSON, P.J. & RODBARD, D. (1983). Number of receptor sites from Scatchard and Klotz graphs: A constructive critique. *Science*, **220**, 979–981.

NOACK, E., FELGENTRÄGER, J. & ZETTNER, B. (1979). Changes in myocardial Na and K content during the development of cardiac glycoside inotropy. *J. mol. cell. Cardiol.*, **11**, 1189–1194.

NOEL, F. & GODFRAIND, T. (1984). Heterogeneity of ouabain specific binding sites and ($Na^+ + K^+$)-ATPase inhibition in microsomes from rat heart. *Biochem. Pharmac.*, **33**, 47–53.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *N.Y. Acad. Sci., U.S.A.*, **51**, 660–672.

SCHWARTZ, A., LINDENMAYER, G.E. & ALLEN, J.C. (1975). The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. *Pharmac. Rev.*, **27**, 3–134.

SILBERGELD, E.K. (1981). Erythrosin B is a specific inhibitor of high affinity ^{3}H-ouabain binding and ion transport in rat brain. *Neuropharmac.*, **20**, 87–90.

SWANN, A.C. (1982). Brain (Na^+-K^+)-ATPase: Biphasic interaction with erythrosin B. *Biochem. Pharmac.*, **31**,

2185–2190.

SWEADNER, K.J. (1979). Two molecular forms of (Na^+-K^+)-stimulated ATPase in brain. *J. biol. Chem.*, **254,** 6060–6067.

WALLICK, E.T., LANE, K.L. & SCHWARTZ, A. (1979). Biochemical mechanism of the sodium pump. *A. Rev. Physiol.*, **41,** 397–411.

WELLSTEIN, A. & PALM, D. (1984). Theory of ligand-receptor interactions – Evidence for more than one site. *Basic Res. Cardiol.*, **79** (Suppl), 9–15.

WERDAN, K., KRAWIETZ, W.E. & ERDMANN, E. (1983). Cardiac glycoside receptors in cultured heart cells: Classification of different receptor subtypes with respect to ouabain binding and coupling sodium pump inhibition. *J. mol. cell. Cardiol.*, **15** (Suppl 2), 53.

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