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BINDING OF CARDIAC GLYCOSIDES TO ISOLATED JEJUNAL BRUSH BORDERS FROM RAT AND GUINEA PIG AND THEIR INFLUENCE ON MEMBRANE PHOSPHATASE SYSTEMS*

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Abstract—The amount of cardiac glycosides tightly bound isolated jejunal brush borders of rat and guinea pig decreases in the following order: peruvosid, digitoxin, proscillaridin, digoxin and ouabain. In rat and guinea pig the *p*-nitrophenylphosphatase (*p*-NPPase) and the Na,K-ATPase of the brush borders are about five times more sensitive to digitoxin than to ouabain. In brush borders of both species the sensitivity of the Na,K-ATPase to cardiac glycosides is more than 100 times higher than that of the *p*-NPPase. In the guinea pig the Na,K-ATPase of the brush borders is 70 times more sensitive to cardiac glycosides than in rat.

COMPARATIVE studies on the intestinal absorption of cardiac glycosides showed differences between several glycosides as well as species differences.[1–4] A correlation exists between the amount of glycosides taken up in the mucosal tissue and the absorption. Poorly absorbed glycosides like the highly polar ouabain are also poorly taken up by the mucosal tissue. Accordingly the higher absorption of the more apolar digitoxin is paralleled by a higher uptake into the tissue.[3] The question arises, whether the luminal membranes of the intestinal epithelium, i.e. the brush borders, already show differences in binding properties. Therefore, the binding of ouabain, digitoxin, digoxin, proscillaridin and peruvosid to isolated jejunal brush borders of rat and guinea pig was measured.

From earlier experiments[5] it is known, that cardiac glycosides inhibit the absorption of calcium, glucose, sodium and water in isolated loops of guinea pig and that different glycosides show different inhibitory activity. It is generally assumed, that the Na,K-ATPase is responsible for transport processes across the mucosal epithelium. Therefore it was tested, whether differences in binding of cardiac glycosides to brush borders are related to differences in inhibitory activity of these glycosides on the Na,K-ATPase of the brush borders. Since brush borders are known to contain a second membrane phosphatase system, the p-NPPase, which also can be influenced by cardiac glycosides,[6] the investigations were extended to this enzyme.

METHODS AND MATERIALS

Female wistar rats (150–200 g; from R. Reupohl, Lage/Lippe) and female guinea pigs (250–300 g; from E. Stock, Gelnhausen) have been used unfasted. Nutrition has been "ssniff" by G. Plange (Soest).

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[^{3}H]ouabain, [^{3}H]digitoxin and [^{3}H]digoxin (NEN-Corp. Boston, U.S.A.), [^{3}H]digitoxin and [^{3}H]peruvosid (E. Merck AG, Darmstadt) and [^{3}H]proscillaridin (Knoll AG, Ludwigshafen) were used with an appropriate amount of carrier glycoside. In all experiments analytical grade chemicals were used. Tris–ATP, tris–ADP and tris-*p*-nitrophenylphosphat were prepared as described by Clausen and Formby.[7]

Preparation of brush borders

Isolated jejunal brush borders were prepared from the proximal 30 cm of the small intestine. Since all attempts to separate the fibrillous components from the microvillous structure membrane led to a loss of the typical microvillous structure of the membrane[8] the method of Forstner *et al.*[9] has been used for the isolation of intestinal brush border fraction which contains the microvillous membranes together with parts of the terminal web.

This method combines repeated homogenization of mucosal scrapings in 5 mM EDTA solution and centrifugation[10] and the purification of the brush borders by treatment with saline buffer and passage through a pad of glass wool.[11] The saline buffer contained 90 mM LiCl and 0·8 mM EDTA pH 7·4. Each step of the preparation and the purification of the brush borders was monitored in a phase contrast microscope. The degree of the purification has been proved by means of electron microscopy.

Protein was determined according to Lowry *et al.*[12] One ml brush border preparation was adjusted to contain 0·7–1·4 mg protein.

Measuring of radioactivity

The [^{3}H]activity bound to the sedimented brush borders was extracted with methanol, diluted in dioxan-type scintillation liquid and measured in a scintillation counter. By means of carrier analysis it was shown in thin layer chromatography that the activity represented the original glycosides (for details see Ref. 3).

Determination of p-*nitrophenylphosphatase activity*

The *p*-nitrophenylphosphatase (*p*-NPPase, EC 3.1.3.1) was determined by measuring the rate of release of *p*-nitrophenol by a modification of the method of Fujita *et al.*[13] and Boyd *et al.*[6] The incubation volume of 1·1 ml contained 0·1 ml brush border preparation (70–140 μg protein), 1·0 ml substrate (5 mM tris-*p*-NPP) + histidin imidazol–HCl buffer (pH 6·8; 50 mM) + 5 mM $MgCl_2$ + 10 mM KCl + 0·25 mM EDTA. After a 30-min incubation at 37° the reaction was stopped with 0·2 ml 30% TCA and *p*-NP absorbance was measured at 405 nm after adding 4 ml 0·15 M NaOH. Specific *p*-NPPase activity is expressed as μmoles *p*-NP released per mg protein and per 30 min. In respect to the solubility of the glycosides the suspension medium contained ethanol in a final concentration of 5 vol. %. In preceding experiments it was shown that ethanol partly (by about 20 per cent) inhibits the *p*-NPPase, but it does not alter on principle the ouabain inhibition.[13,14]

Determination of adenosinetriphosphatase activity

The adenosinetriphosphatase (ATPase, EC 3.6.1.3 and EC 3.6.1.4) was determined by measuring the rate of release of inorganic phosphate (P_i) by a modification of the method of Matsui and Schwartz[15] and of Fiske and Subbarow.[16] The incubation volume of 1·0 ml contained 50 mM tris–HCl pH 7·4, 5 mM tris–ATP, 5 mM $MgCl_2$,

100 mM NaCl, 10 mM KCl, 0·25 mM EDTA and brush border preparation (25–35 μg protein). After 3 min preincubation at 37° the reaction was started by the addition of ATP for an incubation period of 15 min. ATP hydrolysis in control tubes without enzyme or substrate or incubation was determined simultaneously. The reaction was stopped by adding 2 ml of ice-cold 10% TCA. Specific ATPase activity is expressed as μmoles P_i liberated per mg protein and per min. The amount of substrate hydrolysed was linearly related to the time of incubation. ATP was three times faster hydrolysed than ADP. In respect to the solubility of the glycosides the suspension medium contained ethanol in a final concentration of 1 vol. %. Preceding experiments showed that in this concentration ethanol reduced the Na, K-ATPase activity by 12% only and did not change the specific activity of Mg-ATPase. Apparently the sensitivity of the Na, K-ATPase of brush borders to ethanol was lower than that of brain tissue.[14]

RESULTS

(a) *Binding of cardiac glycosides to brush borders*

In order to investigate the binding of cardiac glycosides, isolated brush borders in Krebs-bicarbonate buffer pH 7·4 were preincubated 5 min in a waterbath at 37° (0·7–1·4 mg protein/ml). After the addition of a tritiated glycoside the suspension was incubated for 10 min under gentle shaking in a waterbath. (Previous experiments showed that the binding was completed within 3 min.) After the incubation period the tubes were chilled by ice and centrifuged for 3 min at 3000 *g* at 0°. The supernatant was discarded. The sedimented brush borders were five times resuspended in 5 ml ice-cold isotonic KCl solution and centrifuged for 3 min at 3000 *g*, the supernatant being discarded. After these five washings the [^{3}H]activity bound to the sedimented brush borders was measured.

TABLE 1. AMOUNT OF CARDIAC GLYCOSIDES TIGHTLY BOUND TO BRUSH BORDERS OF RAT AND GUINEA PIG

	Relative amount of glycosides tightly bound to brush borders of	
	Rat	Guinea pig
Ouabain	1·0	1·0
Digoxin	1·3 ± 0·11	1·2 ± 0·18
Proscillaridin	55·8 ± 0·73	54·4 ± 1·0
Digitoxin	72·0 ± 0·71	68·0 ± 5·2
Peruvosid	107·0 ± 18·5	90·0 ± 1·6

Incubation volume of 1·5 ml Krebs-bicarbonate buffer pH 7·4, containing brush borders (0·7–1·4 mg protein). 10^{-5} M glycoside. Five min preincubation, 10 min incubation at 37° under gentle shaking. Determination of [^{3}H]activity in sedimented brush borders after five washings in 5 ml ice-cold isotonic KCl solution.
$n = 4$–8.
$P < 0{\cdot}01$ (paired observations).
Amount of ouabain bound in pmoles × mg protein^{-1} ± S.D.
Rat = 5·77 ± 0·29 = 1.
Guinea pig = 5·65 ± 0·35 = 1.

The bound amount after five washings will be referred to as the *tightly bound* amount of the glycosides. This tightly bound amount of the glycosides, of course, does not represent the situation in distribution equilibrium, but it indicates clearly differences in the tightness of the binding of the glycosides to the brush borders.

The following glycosides were compared in a final concentration of 10^{-5} M: ouabain, digitoxin, digoxin, proscillaridin and peruvosid. The binding of the glycosides varies up to 100-fold (Table 1).

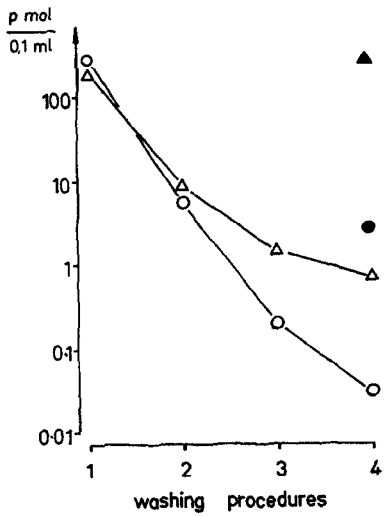

FIG. 1. Concentration of glycosides after washing procedures in the supernatants (open symbols) and in the sediment after the 4th washing (filled symbols). △ = digitoxin, ○ = ouabain. $n = 2$. Incubation volume of 1·5 ml Krebs-bicarbonate buffer pH 7·4 containing brush borders (0·7–1·4 mg protein). 10^{-5} M glycoside. Five min preincubation, 10 min incubation at 37° under gentle shaking. Determination of [^{3}H]activity in sedimented brush borders after four washings in 5 ml ice-cold isotonic KCl solution.

As it would be expected after subsequent washing procedures the concentration of a glycoside which is bound more tightly to the brush borders, decreases in the supernatant fluid at a slower rate, than the concentration of a less tightly bound glycoside. Consequently the concentration of the less tightly bound ouabain decreases at a faster rate than that of the more tightly bound digitoxin (Fig. 1).

The tightly bound amount of digitoxin in rat brush borders is linearly proportional to the concentration (Fig. 2).

For information on the *binding in equilibrium* experiments were carried out in which after the incubation with 10^{-5} M tritiated digitoxin or ouabain 1 ml of the brush border suspension was pipetted on a millipore filter (mean diameter of pores = 3 μm). The [^{3}H]activity retained in the brush borders on the filter was measured. The millipore filter method renders inevitably values which are somewhat too high due to the residue of incubation medium between the brush borders.

The amount of digitoxin bound to the brush borders of rat was 3·15 nM/mg protein (S.D. $\pm$ 0·09, $n = 6$), while the content of ouabain, 0·073 nM/mg protein (S.D. $\pm$ 0·0015, $n = 6$), was about 40 times smaller (for comparison: 70 times in tightly bound amounts, see filled symbols, Fig. 1). As it would be expected the difference between

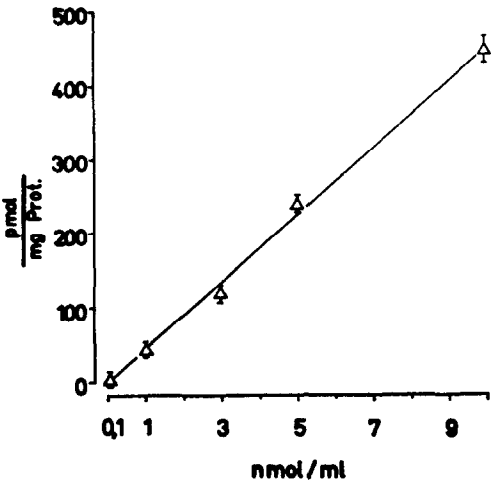

FIG. 2. Concentration dependence of the tightly bound amount of digitoxin in rat brush borders. △ = digitoxin. Incubation volume of 1·5 ml Krebs-bicarbonate buffer pH 7·4 containing brush borders (0·7–1·4 mg protein). Five min preincubation. Ten min incubation at 37° under gentle shaking. Determination of [^{3}H]activity in sedimented brush borders after five washings in 5ml ice-cold isotonic KCl solution. n = 6–8 ± S.D.

digitoxin, the glycoside which is tightly bound to the brush borders, and ouabain, the glycoside which can be removed easily by washing, is smaller in distribution equilibrium.

(b) p-*NPPase activity and inhibition by cardiac glycosides*

The specific activity of *p*-NPPase was in rat 5·6 (S.D. ± 0·7) and in guinea pig 2·1 (S.D. ± 0·1) μmoles per mg protein and 30 min.

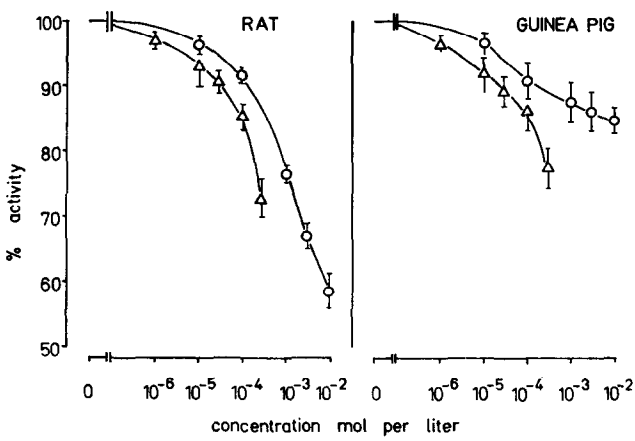

FIG. 3. Inhibition of *p*-nitrophenylphosphatase by cardiac glycosides in brush borders of rat and guinea pig. △ = digitoxin, ○ = ouabain. Incubation volume of 1·1 ml contained 0·1 ml brush border preparation (70–140 μg protein), 1·0 ml substrate (5 mM tris-*p*-NPP + histidin imidazol buffer pH 6·8, 50 mM + 5 mM $MgCl_2$ + 10 mM KCl + 0·25 mM EDTA + glycosides as indicated). Thirty min incubation at 37°. Specific activities in μmoles *p*-NP per mg protein and 30 min.
Rat: 100 = 5·6 (S.D. ± 0·7, n = 4).
Guinea pig: 100 = 2·1 (S.D. ± 0·1, n = 4).

The sensitivity of the *p*-NPPase to digitoxin was generally higher than to ouabain (Fig. 3). 3×10^{-4} M digitoxin inhibit the *p*-NPPase of both rat and guinea pig brush borders by 20–25 per cent. A six times higher concentration of ouabain is needed for approximately the same degree of inhibition in rat brush borders. This is a concentration which in guinea pig brush borders suffices for an inhibition of 15 per cent only. Apparently the sensitivity of the *p*-NPPase of guinea pig is much lower than that of rat.

(c) *ATPase activity and inhibition by cardiac glycosides*

The specific activity of the Mg-ATPase and of the Na, K-ATPase in brush borders of rat and guinea pig jejunum are shown in Table 2.

TABLE 2. SPECIFIC ACTIVITY OF Mg-ATPase AND Na,K-ATPase IN BRUSH BORDERS OF RAT AND GUINEA PIG

	Specific activity			
	Rat		Guinea pig	
	μmoles P_i / mg prot. × min	% of total	μmoles P_i / mg prot. × min	% of total
Mg-ATPase	0·78	60	0·42	75
Na,K-ATPase	0·52	40	0·14	25

Incubation volume of 1·0 ml contained 50 mM tris–HCl pH 7·4, 5 mM tris–ATP, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0·25 mM EDTA and brush border preparation (25–35 μg protein). Three min preincubation and 15 min incubation at 37°. Reaction started by adding ATP, stopped by adding 2 ml ice-cold 10% TCA. $n = 20$, S.D. = $\pm 0{\cdot}07$.

It is difficult to compare these results with data of other authors, because the test conditions usually differ in more than one parameter. The following data have been converted to comparable dimensions (μmoles P_i per mg protein and min). Forstner *et al.*[9] found in rat small intestinal brush borders a specific activity of the Mg-ATPase of 0·275, Berg and Szekerczes[17] found in rat small intestinal microvilli fraction a specific activity for Mg-ATPase of 0·22 and for Na,K-ATPase of 0·133. Taylor[18] found in guinea pig small intestinal mucosa a specific activity for Mg-ATPase of 0·125 and for Na,K-ATPase of 0·105. Robinson[19] found a microsomal Na,K-ATPase of rat small intestine a S.A. of 0·3, but since Rostgaard and Møller[20,21] show edthat 75 pe rcent of a "microsomal" Na,K-ATPase of kidney cortex is actually derived from the microvillous membrane, this might also be true for the microsomal Na,K-ATPase of the intestinal epithelium.

The influence of different concentrations of ouabain and digitoxin on the Na,K-ATPase activity is shown in Fig. 4. Fifty per cent inhibition of the Na,K-ATPase is produced by 8×10^{-7} M digitoxin and by 3×10^{-6} M ouabain in guinea pig, while in rat for 50 per cent inhibition 5×10^{-5} M digitoxin and 10^{-4} ouabain is needed. In other words: In both species the sensitivity of the Na,K-ATPase to digitoxin is about four times higher than that to ouabain. The Na,K-ATPase of guinea pig is about 70 times more sensitive to both glycosides than that of rat.

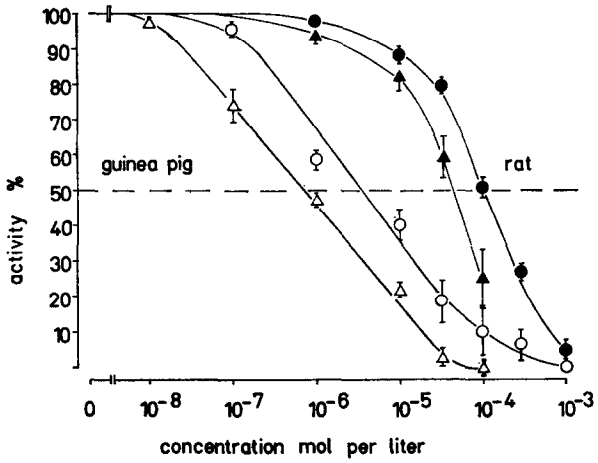

FIG. 4. Inhibition of Na,K-ATPase by glycosides in rat and guinea pig. △ = digitoxin, ○ = ouabain. Incubation volume of 1·0 ml contained 50 mM tris–HCl pH 7·4, 5 mM tris–ATP, 5 mM $MgCl_2$ 100 mM NaCl, 10 mM KCl, 0·25 mM EDTA, brush border preparation (25–35 μg protein) and glycosides as indicated. Three min preincubation and 15 min incubation at 37°. Reaction started by adding ATP, stopped by adding 2 ml of ice-cold 10% TCA. Specific activity in μmoles P_i per mg protein and min.

Rat: 100 = 0·42.
Guinea pig: 100 = 0·14.
S.D. ≤ ± 0·07, n = 6–8.

DISCUSSION

The first question formulated in the introduction was: Whether a correlation exists between the different absorption rates of cardiac glycosides and their uptake into the brush border membrane. The answer given by the results is no. Although brush borders from *guinea pig* take up about 70 times more digitoxin than ouabain, the absorption rate of these two glycosides is nearly equal in isolated segments of the jejunum.[3] Only in rat a qualitative correlation could be stated where the absorption rate of digitoxin is about seven times higher than that of ouabain.

In regard to the different properties of the two subjects compared, the binding of glycosides in brush borders and their transcellular movement, the result does not surprise. For the penetration of the luminal membrane, i.e. the brush border membrane, the lipid solubility e.g. could be mainly the limiting factor, while the transfer across the different intracellular compartments can be influenced by many other factors, e.g. the capability of the glycosides to react with proteins.

If one assumes that the lipid solubility is the determining factor for the uptake and binding of cardiac glycosides in brush borders a correlation to the polarity would be expected. The order of the glycosides, arranged according to decreasing tightness of binding to the brush borders, is for rat as well as for guinea pig: peruvosid, digitoxin, proscillaridin, digoxin and ouabain.

In regard to these data it seems allowed to state, that in general the binding of less polar glycosides to isolated brush borders is higher than that of more polar glycosides, but it does not follow strictly the order in polarity. Forth *et al.*[3] found by means of thin layer chromatography (mobile phase: water-saturated methyl-ethyl-keton) the

following order in increasing polarity: digitoxin, peruvosid, proscillaridin, digoxin and ouabain. Greenberger *et al.*[4] studied the chloroform–water partition coefficients and found the following order of increasing polarity: digitoxin (89·67), digoxin (0·998), proscillaridin (0·465) and ouabain (0).

In view of the lacking of a strict correlation it should be taken into account that on one side the models for measuring the lipid solubility are not equal to the membrane lipids and on the other side the glycosides may interact also with other parts of the brush borders, e.g. the glycocalix, the membrane proteins and specific binding sites of membrane phosphatases.

The answer to the second question, whether a correlation exists between the binding of the glycosides to brush border membranes and the inhibition of membrane phosphatases, is also no. The Na,K-ATPase of brush borders of guinea pig is about 70 times more sensitive to digitoxin and to ouabain than that of rat, while in both species the tightly bound amount of the two glycosides is nearly the same. Furthermore the inhibitory activity of digitoxin in guinea pig is only about five times higher than that of ouabain in spite of the fact that the tightly bound amount of digitoxin in brush borders was about 70 times higher. Consequently only the affinities of the glycosides to the Na,K-ATPase but not to the non-specific binding sites in the membrane seems to differ. Therefore, the conclusion has to be drawn that such a correlation is not allowed because the data of these binding experiments represent an overall binding, making it impossible to discriminate between specific binding, e.g. binding to special receptors in the phosphatase systems, and non-specific binding to other sites in the membrane.

Finally, if the Na,K-ATPase is important for transport processes across the mucosal epithelium, it should be worthwhile to look for a correlation between the inhibitory activity of cardiac glycosides on the Na,K-ATPase of isolated brush borders and on the absorption of glucose in isolated jejunal segments. For a 50 per cent inhibition of glucose absorption in guinea pig about a five times higher concentration of ouabain (5×10^{-5} M) is needed than of digitoxin (10^{-5} M).[5] This ratio agrees with the observation, that also for a 50 per cent inhibition of Na,K-ATPase in brush borders of guinea pig about a five times higher ouabain concentration is needed. The concentration of the two glycosides, which inhibit the Na,K-ATPase of the highly purified brush borders are about ten times lower than those which inhibit the glucose transport in gut segments. This difference should not surprise in regard to the very different conditions, e.g. the possibilities for binding in compartments of the intestinal wall which do not take part in active transport.

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