Measurement of specific [^3H]-ouabain binding to different types of human leucocytes

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1 We have studied the specific binding of [^3H]-ouabain to intact mononuclear leucocytes (82% lymphocytes) and polymorphonuclear leucocytes.

2 In both types of cells [^3H]-ouabain binding was saturable, confined to a single site of high affinity, slow to reach equilibrium, slow to reverse, temperature-dependent, competitively antagonized by potassium, and facilitated by the presence of divalent cations.

3 The equilibrium dissociation constants were 2.4 ± 0.7 nmol/l (polymorphs) and 2.4 ± 0.4 nmol/l (mononuclear cells) (NS).

4 The values of maximal specific ouabain binding, measured by Scatchard analysis of concentration *vs* binding curves (B_{max}), were 33.9 ± 6.0 fmol/ 10^6 cells (polymorphs) and 59.3 ± 11.6 fmol/ 10^6 cells (mononuclear cells) ($P < 0.02$). The corresponding numbers of sites per cell were 20415 ± 3616 and 35712 ± 6986 respectively ($P < 0.02$). When the numbers of binding sites were expressed per square micron of cell surface area the difference between the two cell types was proportionately greater (83 and 186 sites per μ^2 respectively).

5 We conclude that the [^3H]-ouabain binding sites on mononuclear and polymorphonuclear leucocytes are similar in nature, but different in both number and density on the cell surface. Measurements of B_{max} in mixed cell populations should therefore take account of cell type as well as cell size and number.

Keywords leucocytes polymorphonuclear mononuclear cardiac glycosides receptors

Introduction

In recent years there has been increasing interest in delineating *in vitro* abnormalities of cation transport in human erythrocytes and leucocytes in different diseases, such as chronic renal failure, hypertension, and manic-depressive psychosis. Much of the work on human leucocytes has been carried out on mixed populations of the different types of leucocyte, and since the different types of cells have different functions physiologically, we decided to compare cation transport mechanisms in separate types of cell. In this paper we report the results of our investi-

gations of specific cardiac glycoside (ouabain)-binding in mononuclear and polymorphonuclear leucocytes.

Methods

Isolation of intact mononuclear and polymorphonuclear leucocytes from whole blood

Suspensions of intact mononuclear (MN) and polymorphonuclear (PMN) leucocytes were pre-

pared from whole blood (40–120 ml) by centrifugation on a discontinuous density gradient of Percoll (Harbeck *et al.*, 1982; Giudicelli *et al.*, 1982). Whole venous blood, anticoagulated with 1% EDTA, was diluted with an equal volume of phosphate-buffered saline solution (125 mmol/l NaCl, 13.8 mmol/l Na_2HPO_4 , 3.2 mmol/l NaH_2PO_4 , 0.34 mmol/l EDTA), and mixed with dextran (M.Wt 250,000 daltons, final concentration 1.2% w/v). This solution was left standing for 30 min at room temperature to allow the erythrocytes to sediment. The supernatant was removed and centrifuged at 200 g for 10 min at 5°C. The resulting cell pellet was resuspended in a small volume of phosphate-buffered saline, layered onto a discontinuous density gradient of Percoll, and centrifuged at 400 g for 30 min at 5°C. The gradient was prepared with three layers of Percoll (1.06, 1.08 and 1.09 g/ml) so that the different cells were concentrated between different layers (mononuclear leucocytes between the two upper layers, and polymorphonuclear leucocytes between the two lower layers). Cells were harvested by aspiration into a syringe and were washed three times (resuspended and centrifuged at 200 g for 15 min) in the incubation medium.

The purity of the final cell suspension was tested by inspecting stained smears. Polymorphonuclear and mononuclear cells were differentiated by staining with Diff-Quick (Harleco, Belgium); lymphocytes and monocytes were differentiated by staining with a non-specific esterase (Yam *et al.*, 1971). The mononuclear cell suspension contained 95% (86–98%; $n = 8$) mononuclear cells of which 82% (77–85%; $n = 4$) were lymphocytes and the remainder monocytes. The polymorphonuclear cell suspension contained 96% (89–99%; $n = 8$) granulocytes, i.e. neutrophils, eosinophils and basophils, the principal contaminant being lymphocytes (percentages given as the median and range). This method of separation yielded 30–50% of the leucocytes present in whole blood.

In some experiments mononuclear leucocytes were prepared using Ficoll, in a modified version of the method described by Böyum (1968). Whole blood (three parts), anticoagulated with heparin (4 units/ml blood), was diluted with RPMI 1640 culture medium (one part) (Gibco, Europe), layered onto Ficoll/sodium diatrizoate (density, 1.077 g/ml; one part) and centrifuged at 400 g for 30 min at 30°C. Mononuclear cells were concentrated at the top of the Ficoll layer and were harvested by aspiration into a syringe and washed three times (resuspended and centrifuged at 200 g for 15 min) in the incubation medium.

In contrast to the separation method using

Percoll, this separation method gave a high yield (approximately 70% of the leucocytes in whole blood), but with appreciable contamination (50–100%) with platelets. The nucleated cells were 98% (94–100%; $n = 6$) mononuclear leucocytes, of which 83% (79–84%; $n = 4$) were lymphocytes and the remainder monocytes.

The experiments reported here were carried out using the Percoll method to establish the characteristics of binding of ouabain to leucocytes. Measurements of the numbers of binding sites and the affinities of those sites were then carried out using both methods under standard conditions, and the results are reported below.

Measurement of [^3H]-ouabain binding to intact leucocytes

Intact leucocytes were washed three times and then resuspended in a freshly made potassium-free Ringer solution (Aronson *et al.*, 1977) which was used as the incubation medium. The final cell density was adjusted to be between 1.5×10^6 and 3×10^6 cells/ml. Specific binding was found to be linearly related to cell density up to 6×10^6 cells/ml. Cell viability was assessed by Trypan blue staining and was always greater than 97% at the end of the isolation procedure, and greater than 95% at the end of a 6-h incubation.

[^3H]-ouabain (100 μl , specific activity 14–18 Ci/mmol), diluted with the incubation medium, was added to each Eppendorf incubation tube (final concentration 1–25 nmol/l), together with either 100 μl of incubation medium alone or 100 μl of the incubation medium containing digoxin (final concentration 5 $\mu\text{mol/l}$), in order to identify total and non-specific binding respectively. Incubations were begun by adding 800 μl of the cell suspension to each of the tubes, which were then capped, vortex-mixed, and placed in a shaking water-bath at 37°C. Incubations were continued in duplicate or triplicate for up to 6 h at 37°C and terminated by centrifugation at 6500 g for 3 min in an Eppendorf bench centrifuge. Of the supernatant 100 μl was collected and counted for radioactivity to determine the free concentration of [^3H]-ouabain; the remaining supernatant was discarded. In order to remove any unbound ligand the cell pellets were rinsed three times with 0.5 ml of the incubation medium, and the Eppendorf tubes were then inverted and allowed to drain for at least 30 min. Finally, 500 μl of distilled water was added to each tube and the cell pellets were disrupted by ultrasonication using a Dawe soniprobe (intensity 3–4 for 6 s). A 400 μl aliquot was taken from each tube, placed in a plastic vial with 5 ml of PCS liquid scintillant, and counted for 10 min in a liquid scintillation

counter. The amount of [^3H]-ouabain bound was expressed in units of fmol/ 10^6 cells.

Equilibrium binding data analysis

Specific binding was calculated by subtracting non-specific binding from total binding. Total binding did not exceed 5% of the total amount of radioactivity present in any incubation. The maximum binding capacity (B_{max}) and the equilibrium dissociation constant (K_D) were determined by Scatchard analysis (Scatchard, 1949) of the specific equilibrium binding data for five free concentrations of [^3H]-ouabain.

The second-order association rate constant (k_{ass}) was measured by determining the binding of [^3H]-ouabain at several time intervals up to 8 h in the presence of 2.8, 10 and 25 nmol/l [^3H]-ouabain, and calculated using the method of Williams & Lefkowitz (1978).

The first-order dissociation rate constant (k_{diss}) was measured by first allowing maximum binding to occur over a 2-h incubation period in [^3H]-ouabain (25 nmol/l), and then measuring the binding of [^3H]-ouabain for 16 h thereafter following the addition of excess unlabelled

digoxin (5 $\mu\text{mol/l}$). The dissociation rate constant (k_{diss}) was taken to be the slope of the line relating $\log_e$ [^3H]-ouabain to time, after correction for the [^3H]-ouabain binding found after addition of digoxin-free incubation medium as a control.

Subjects and statistical analyses We studied 16 healthy subjects, aged 25–65 years (10 men). Statistical comparisons were by paired and unpaired two-tailed *t*-tests, with Cochran's modification if variances were inhomogeneous.

Results

We studied [^3H]-ouabain binding simultaneously in MN and PMN leucocytes prepared from the same sample of blood.

Specificity of binding Specific [^3H]-ouabain binding (that which could be inhibited by the presence of digoxin, 5×10^{-6} mol/l) constituted 80–99% of total binding. A digoxin concentration of 5×10^{-6} mol/l was chosen on the basis of initial experiments which showed that the total binding of [^3H]-ouabain fell sharply as the con-

Figure 1 The inhibition of total [^3H]-ouabain binding to (a) intact PMN and (b) intact MN leucocytes by digoxin.

Specific binding was measured after incubating leucocytes with 15 nmol/l [^3H]-ouabain in the presence of different concentrations of digoxin; each point represents the mean of four replicates. On the basis of this and other similar experiments, a concentration of 5 $\mu\text{mol/l}$ digoxin was chosen to define specific [^3H]-ouabain binding.

centration of digoxin was increased from 10^{-9} to 10^{-6} mol/l, but remained almost constant as the concentration of digoxin was increased further (Figure 1).

The dissociation constants (K_i) for the inhibition of [3 H]-ouabain binding by digoxin were calculated according to the formula described by Cheng & Prusoff (1973) and were 4.6×10^{-9} mol/l and 4.3×10^{-9} mol/l for MN and PMN respectively (mean of three estimates).

Kinetics of specific [3 H]ouabain binding

Association The rate of specific [3 H]-ouabain binding to MN and PMN leucocytes was dependent on the concentration of free [3 H]-ouabain (Figure 2) and was slow (apparent half-time approximately 1 h at a free ouabain concentration of 10×10^{-9} mol/l). There was a tendency for specific [3 H]-ouabain binding to fall after incubation for 6–8 h. This made it difficult to determine whether true equilibrium was reached at the lowest concentrations of [3 H]-ouabain (see discussion below).

Dissociation The dissociation of [3 H]-ouabain from the [3 H]-ouabain-leucocyte complex was very slow (Figure 3) with a half-time of 7.3 h for MN leucocytes and 8.0 h for PMN leucocytes (Table 1).

The second-order association rate constants (k_{ass}) and the first-order dissociation rate constants (k_{diss}) are given in Table 1. The dissociation equilibrium constants (K_D) derived from these rate constants were 1.2×10^{-9} mol/l and 1.0×10^{-9} mol/l for MN and PMN leucocytes

respectively, and are somewhat lower than those derived from Scatchard analysis of equilibrium binding data (see discussion below).

Equilibrium binding characteristics for specific [3 H]-ouabain binding The specific binding of [3 H]-ouabain to intact human leucocytes was saturable, and Scatchard analysis of equilibrium binding data produced correlation coefficients close to unity, implying that binding was confined to a single site (Figure 4). In a group of eight healthy volunteers (aged 24–36 years; six men) there were significantly more binding sites on the mononuclear cells compared with the polymorphonuclear cells (Table 1). The K_D for binding to MN cells was slightly lower than for PMN cells, but we consider the difference (which is statistically significant, $P < 0.05$, by a paired *t*-test) to have occurred by chance, for reasons discussed below.

The specific maximum binding obtained by incubating cells with 25×10^{-9} mol/l [3 H]-ouabain (B_{max}) was very close to the value for B_{max} obtained by Scatchard analysis (Table 1), and was used as a convenient method for estimating B_{max} with a small volume of blood (30 ml). This method gave reproducible results with inter-assay coefficients of variation, determined by measuring specific binding at 25×10^{-9} mol/l [3 H]-ouabain in the same subject on five consecutive days, of 11.3% for MN leucocytes and 12.3% for PMN leucocytes.

Effects of other variables on specific [3 H]-ouabain binding

Potassium Potassium (1 and 2 mmol/l) in-

Figure 2 The association time course for specific [3 H]-ouabain binding to (a) intact PMN and (b) intact MN leucocytes.

Specific [3 H]-ouabain binding was measured at three different concentrations of free [3 H]-ouabain (2.8 ($\square$), 10 ($\blacktriangle$) and 25 ($\circ$) nmol/l) using a wide range of incubation times (5 min to 20 h). Each point is the mean of three or four replicates; all of these experiments were carried out using blood from the same subject.

Figure 3 The dissociation time-course for specific [^3H]-ouabain binding to intact MN and PMN leucocytes.

Intact leucocytes were preincubated with 25 nmol/l [^3H]-ouabain for 2 h and then [^3H]-ouabain binding was measured at various times from 0 to 16 h after the addition of either digoxin, final concentration 5 $\mu\text{mol/l}$ (▲) or incubation medium alone (●). Non-specific ouabain binding was measured in parallel incubations and the results were expressed as a percentage of the specific [^3H]-ouabain binding present at the moment the digoxin was added. Each point is the mean of three separate observations.

Table 1 A comparison of the characteristics of [^3H]-ouabain binding to intact PMN and MN leucocytes isolated by centrifugation of Percoll and intact MN leucocytes isolated by centrifugation on Ficoll/sodium diatrizoate

	Isolation method		
	PMN	MN	MN
<i>Binding kinetics</i> (mean of three experiments in one individual)			
Association rate constant (k_{ass}) ($\text{nm}^{-1} \text{h}^{-1}$)	0.087	0.079	0.065
Association half-time (10 nM) (h)	0.94	1.01	1.12
Dissociation rate constant (k_{diss}) (h^{-1})	0.087	0.095	0.100
Dissociation half-time (h)	8.0	7.3	7.0
K_D ($k_{\text{ass}}/k_{\text{diss}}$) (nM)	1.0	1.2	1.5
<i>Equilibrium binding characteristics</i> (mean $\pm$ s.d.)			
K_D (nM)	$n = 8$ 2.42 ± 0.7	$n = 8$ $1.96 \pm 0.42^{*†}$	$n = 16$ 2.39 ± 0.51
B_{max} (fmol/ 10^6 cells)	33.9 ± 6.0	$59.3 \pm 11.6^{**}$	66.4 ± 13.3
Number of sites per cell	20415 ± 3616	$35712 \pm 6986^{**}$	39987 ± 8009
Estimated no. sites per μ^2 cell surface ¹	83	186	208
B_{max} (25 nM [^3H]-ouabain) (fmol/ 10^6 cells)	$n = 15$ 31.0 ± 4.5	$n = 15$ 56.7 ± 9.3	$n = 14$ 62.8 ± 11.7

All experiments were carried out on blood obtained from healthy subjects. Values for MN leucocytes isolated by centrifugation on Percoll were compared to those for PMN leucocytes isolated by centrifugation on Percoll using Student's paired t -test (* $P < 0.05$, ** $P < 0.02$) and values for MN leucocytes isolated on Ficoll/sodium diatrizoate were compared to those for MN leucocytes isolated on Percoll using Student's unpaired t -test ($^{\dagger} P < 0.05$).

¹ Estimated from three measurements of cell volume and assuming that each cell is a perfect sphere.

Figure 4 The specific binding of [^3H]-ouabain to (a) intact PMN and (b) intact MN leucocytes from a healthy individual.

Specific binding was determined by incubating intact cells with [^3H]-ouabain (1.5–15 nmol/l) for 6 h at 37°C. Each point is the mean $\pm$ s.e. mean of four replicates. The insets show the same data analysed by Scatchard analysis.

hibited specific [^3H]-ouabain binding, increasing the K_D of ouabain binding without altering the total number of binding sites (not shown), indicating that potassium was acting as a competitive inhibitor. The changes in MN and PMN leucocytes were the same, 1 mmol/l potassium increasing the K_D to 4.6×10^{-9} mol/l and 2 mmol/l potassium increasing it to 7.0×10^{-9} mol/l.

Divalent cations When cells were incubated with [^3H]-ouabain in the absence of calcium and magnesium ions the binding capacity ($B_{\max}$) was not altered but the K_D was increased to 3.5×10^{-9} mol/l in MN leucocytes and 7.2×10^{-9} mol/l in PMN leucocytes (not shown).

Temperature The rate of [^3H]-ouabain binding was temperature dependent, with a Q_{10} (from 27°C to 37°C) for the initial rate of binding of 1.8 and 2.4 for MN and PMN leucocytes respectively (not shown). Binding was not detectable at either 4°C or 47°C.

[^3H]-Ouabain binding to intact mononuclear leucocytes prepared by centrifugation on Ficoll

The characteristics of [^3H]-ouabain binding to intact mononuclear cells prepared by centrifugation on Ficoll/sodium diatrizoate were both qualitatively and quantitatively similar to those of intact mononuclear cells prepared by centrifugation on Percoll (see Table 1). The binding capacity ($B_{\max}$) was not significantly different to that for MN leucocytes isolated by centrifugation on Percoll, but the equilibrium dissociation constant (K_D) was slightly greater ($P < 0.05$).

The inter-assay coefficients of variation for the K_D and $B_{\max}$, determined by Scatchard analysis of specific binding data (at six different concentrations of [^3H]-ouabain) for intact MN leucocytes obtained from the same individual on six separate occasions during a period of 4 weeks, were 11.7% and 8.2% respectively.

Discussion

The binding of cardiac glycosides to Na^+, K^+ -ATPase has been studied extensively in several different tissues and species (see Schwartz *et al.*, 1975), but glycoside binding to intact human leucocytes, particularly polymorphonuclear leucocytes, has not been studied in detail, even though these cells have been used to study functional changes in several disease states (Edmondson *et al.*, 1975; Sewell *et al.*, 1982). In lymphocytes glycoside binding has been shown to be saturable, confined to a single site of high affinity, temperature-sensitive, slow to reach equilibrium, slow to reverse, and competitively inhibited by potassium (Quastel & Kaplan, 1975; Pedersen & Klitgaard, 1983). Our work has confirmed these qualitative observations in intact mononuclear leucocytes (the majority of which were lymphocytes), and has demonstrated the same properties in intact polymorphonuclear leucocytes.

Although there are considerable differences among species, the glycoside receptors from different tissues in the same species tend to have the same affinity for ouabain (Erdmann & Schoner, 1973). The equilibrium dissociation constants

calculated from our data are similar to those found by Erdmann & Hasse (1975) in human erythrocytes (2.8×10^{-9} mol/l) and cardiac membranes (2.8×10^{-9} mol/l). Although other workers have reported values ranging from 2.9 to 7.8×10^{-9} mol/l for human erythrocytes we believe that these figures are based on experiments in which binding was not at equilibrium, and that a value of about 1 to 2×10^{-9} mol/l is a more accurate assessment of the true K_D of glycoside binding to the membranes of intact erythrocytes (see Ford *et al.*, 1979). We believe that the K_D for MN leucocytes isolated on Percoll was by chance lower than the K_D for both PMN leucocytes isolated on Percoll and MN leucocytes isolated on Ficoll, for two reasons: firstly, there was no difference between the K_D for binding to MN leucocytes isolated on Ficoll and that for PMN leucocytes isolated on Percoll, and secondly, the differences were very small (e.g. the 95% confidence limits for the difference between MN leucocytes isolated on Percoll and Ficoll were 0 to 0.86×10^{-9} mol/l).

In contrast to the K_D , the number of glycoside-binding sites per cell varies from tissue to tissue within the same species, although these differences are generally much smaller if the number of binding sites is considered as a function of the surface area of the cell (Baker & Willis, 1972). However, in our experiments PMN leucocytes were found to have significantly fewer binding sites than MN leucocytes in terms of both the number of sites per cell and the number of sites per square micron of cell surface.

As far as the different types of mononuclear cell are concerned, monocytes have a significantly greater number of [3 H]-ouabain binding sites per cell compared with lymphocytes, although the numbers of sites per unit of cell volume or of surface area are similar (Wiley *et al.*, 1979; Pedersen & Klitgaard, 1983). In our experiments the percentage of monocytes in the final mononuclear cell suspensions varied between 15% and 23% and this may have introduced some variability into the measurement of $B_{\max}$, although the inter-assay coefficient of variation was only 8.2%. The mononuclear cell suspension produced by centrifugation of blood on Ficoll/sodium diatrizoate contained a significant number of platelets (50–100% contamination), but since platelets have fewer than 750 [3 H]-ouabain binding sites per cell (Wiley *et al.*, 1979, and personal observations), the resulting error in the measurement of $B_{\max}$ must have been less than 2%. Attempts to purify the cell suspensions by using defibrinated blood greatly reduced the yield of leucocytes and were therefore abandoned.

One of the most striking features of the [3 H]-

Table 2 The equilibrium binding characteristics for specific [3 H]-ouabain binding to intact mononuclear leucocytes

Source	Cells	$B_{\max}$ (sites/cell)	K_D (nM)	(K^+) (mM)	Incubation time (h)	Definition of specific binding
This study	MN (Percoll)	$35,712 \pm 6,986$	1.96 ± 0.42	0	6	5 μ M digoxin
(1)	MN (Ficoll)	$39,987 \pm 8,009$	2.39 ± 0.51	0	6	5 μ M digoxin
	L	125,000	3.3	0	1–33 in culture	Total
			15	5.6	1–33 in culture	Total
(2)	L	$44,200 \pm 9,920$	87	4.0	4	0.1 mM ouabain
(3)	MN	$194,400 \pm 18,060$	68 ± 12	5.0	1.5	0.5 mM ouabain
(4)	L	$43,154 \pm 8,037$	6.8 ± 2.1	0	4	1 mM ouabain
	MN	$75,000 \pm 6,764$	6.3 ± 1.2	0	4	1 mM ouabain

Values are given as the mean $\pm$ s.d. value quoted by the respective authors. L indicates a value for pure lymphocytes and MN indicates a value for a mixture of lymphocytes and monocytes.

References (1) Quastel & Kaplan (1975), (2) Wiley *et al.* (1979), (3) Arnott *et al.* (1982), (4) Pedersen & Klitgaard (1983).

ouabain binding characteristics in these, and other, cells is the very slow rate of both association and dissociation. The potential problems of studying a slowly equilibrating ligand-receptor interaction have been discussed by Arányi (1979, 1980), who points out that if the receptor or receptor-ligand complex are denatured at a significant rate, relative to the rate of receptor-ligand association, true equilibrium cannot be reached. In our experiments it was clear that binding had reached equilibrium at high concentrations of [^3H]-ouabain but we could not be certain that this was the case at the lowest concentrations. Figure 2 shows the association time course for a free concentration of [^3H]-ouabain of 2.8×10^{-9} mol/l; superficially it would appear that equilibrium is reached at about 6 h, but further incubation was associated with a fall in binding, and it seems likely that this fall was due to denaturation of the receptor-ligand complex (even though cell viability as tested by Trypan blue staining was > 90% even after 24 h incubation). It is possible, therefore, that these data show a form of pseudo-equilibrium occurring when the rate at which new receptor-ligand complexes are formed equals the rate at which existing complexes are denatured. This interpretation would also explain why the K_D derived from direct measurements of the association and dissociation rate constants is somewhat lower than the K_D obtained from Scatchard analysis of 'equilibrium' binding data. Scatchard analysis of binding data cannot show evidence of failure to reach equilibrium and if this technique is applied to data which are not obtained at equilibrium it will produce artificially high values for K_D (Arányi, 1979).

In Table 2 the results presented here are compared with those of other investigators, most of whom report higher values for both B_{max} and K_D .

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- In conclusion, we have found no significant differences between mononuclear cells and polymorphonuclear cells in the nature of [^3H]-ouabain binding to intact cells, but the number of sites per cell varies with cell type. This variation is not accounted for by differences in cell size. Our qualitative findings are similar to those previously reported for [^3H]-digoxin binding to human erythrocytes (Ford *et al.*, 1979).
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