THE CHARACTERISTICS OF [³H]-OUABAIN BINDING TO HUMAN LYMPHOCYTES

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- 1 The kinetic characteristics of [³H]-ouabain binding to human lymphocytes and mixtures of mononucleated cells, and the maximum [³H]-ouabain binding capacities of these cells were studied.
- 2 The [³H]-ouabain binding was compatible with a single class of receptors with a high affinity for the drug. No signs of positive or negative cooperativity could be demonstrated.
- 3 In six experiments with pure lymphocyte preparations, the association and dissociation rate constants were $3.08 \pm 0.34 \times 10^4/\text{M/s}$ and $1.58 \pm 0.50 \times 10^{-4}/\text{s}$. The dissociation constant derived from equilibrium studies on lymphocytes was $0.68 \pm 0.21 \times 10^{-8} \text{ M}$, which was identical to that of mononucleated cells.
- 4 In healthy subjects the maximum [³H]-ouabain binding capacities, which reflect the number of sodium/potassium pump sites were 43154 ± 8037 molecules/cell ($n = 25$) in lymphocytes and 75474 ± 6764 ($n = 9$) molecules/cell in mixtures of mononucleated cells.
- 5 Direct determination of the [³H]-ouabain binding capacity of lymphocytes can be performed with acceptable accuracy and precision using 30 ml whole blood. Provided high cell purity, this method may be useful, when studying sodium/potassium pump function in clinical settings.

Introduction

Ouabain binds specifically to the Na^+/K^+ ATPase enzyme in cell membranes and inhibits active sodium/potassium transports (Clausen *et al.*, 1974; Schwartz *et al.*, 1975). In extensive investigations (Barnett, 1970; Erdmann *et al.*, 1976; Glynn *et al.*, 1975; Hansen *et al.*, 1971; Jørgensen, 1974) this binding has been demonstrated to occur with a 1:1 molar stoichiometry and it is generally accepted that the number of specifically bound ouabain molecules closely reflects the number of Na^+/K^+ pump units.

Increasing evidence suggests that Na^+/K^+ pump activity and the number of pump units are subject to changes both during physiological conditions and in various disease states. Animal experiments (Gonick *et al.*, 1977; Gruber *et al.*, 1980) and clinical studies (De Wardener *et al.*, 1981) indicate that Na^+/K^+ pump function is regulated by an intrinsic ouabain-like substance and this system may possibly constitute an important link in the delicate regulation of body fluid compartment, sodium balance and blood pressure. Inappropriate high plasma levels of this substance with subsequent depression of pump activity has been proposed as an aetiological factor in essential hypertension (Haddy *et al.*, 1982; Poston *et al.*, 1981). In border-line hypertension (Nielsen *et al.*, (to be published)), sustained hypokalaemia (Erdmann *et al.*, 1977) and during chronic exposition to digitalis glycosides (Boardman *et al.*, 1972; Bonn *et*

al., 1978) or glucocorticosteroids (Kaji *et al.*, 1981) an increase of the number of pump sites has been demonstrated.

Most studies on pump function have used erythrocytic Na^+/K^+ ATPase preparations (Erdmann *et al.*, 1975; Ford *et al.*, 1979; Gardner & Conlon, 1972; Gardner *et al.*, 1973; Kaji *et al.*, 1981). However, this highly specialized cell may be less relevant to study, since it possesses comparatively few pump sites (300/cell) (Erdmann *et al.*, 1975) and is devoid of a DNA-nucleus, which prohibits synthesis of pumps *de novo*. Accordingly, there seems to be a need for a cell type, which constitutes a more appropriate model of the body tissues with regard to Na^+/K^+ pump function.

The purpose of the present investigation was to study the kinetics of [³H]-ouabain binding to human lymphocytes, to measure the number of pump sites in pure lymphocytic preparations and a mixture of mononucleated cells from normal individuals, and to evaluate the practical applicability of this method in clinical settings.

Methods

Materials

[³H]-ouabain (specific activity $\sim 14 \text{ Ci/mmol}$), dis-

solved in ethanol-benzene (9:1 v/v), was purchased from New England Nuclear, West Germany. [^{14}C]-sucrose (specific activity $\sim 15 \text{ mCi/mmol}$) was from Amersham, England. Dibutylphthalate (density ~ 1.044) and dionylphthalate (density ~ 0.98) were obtained from Merch Co., West Germany. Liquid scintillation solution (Picoflour 30) was from Packard Instrument Company, U.S.A. Ficoll-Isopaque, density 1.077, was a product of Pharmacia, Sweden. All chemicals were of analytical grade.

The cells were incubated in potassium-free Krebs-Ringer bicarbonate buffer containing 5 mM glucose and 1% polyvinylpyrrolidone.

Cell preparations

Human peripheral lymphocytes were purified using freshly drawn venous blood from healthy volunteers. The blood was immediately defibrinated by slow agitation with small glass beads in closed glass tubes for 10 min. After dilution with equal volumes of isotonic saline, the lymphocytes were separated by centrifugation on a Ficoll-Isopaque gradient at 300 g for 20 min at room temperature. The cells were then washed twice in potassium-free Krebs-Ringer bicarbonate buffer (KRBB) by alternate suspension and centrifugation at 500 g for 3 min. Cell counts of the original blood samples were performed on a Coulter Counter S $^{\circ}$ while the final cell suspension was counted manually using a Fuchs-Rosenthal counting chamber. This separation method was characterized by a comparatively low yield: 35% ($n = 10$) and a high purity: lymphocytes = 94%, polymorph neutrophils = 3% and monocytes = 2% ($n = 10$). Trypan blue staining revealed a cell viability of more than 97%.

Mononuclear cells were also prepared from peripheral blood using EDTA as anticoagulant. Cell separation and subsequent washing were performed as described above. This method gave a higher yield: 70% ($n = 10$), but lower purity: lymphocytes = 80%, monocytes = 16% and polymorph neutrophils = 4% ($n = 10$).

[^3H]-ouabain binding

Cell incubation took place at 37°C in a shaking water bath using closed polyethylene incubation vials. [^3H]-ouabain was evaporated to dryness and redissolved in potassium-free KRBB. Appropriate volumes of this tracer solution were then added to incubation vials resulting in final [^3H]-ouabain concentrations which spanned from 1×10^{-9} to $3.5 \times 10^{-7} \text{ M}$. At each concentration level, parallel incubation vials containing tracer and 10^{-3} M ouabain were run in order to measure the amount of cellular [^3H]-ouabain uptake which was undisableable in the presence of excess unlabelled drug (non-specific binding). At specified

times, duplicate 300 μl samples of the incubation media were transferred to polyethylene microcentrifuge tubes containing 200 μl dionylphthalate/dibutylphthalate (3:1 v/v). Without delay, these tubes were then centrifuged at 10000 g for 1 min, which caused the lymphocytes to descend through the immiscible oil phase and appear as a thin layer at the bottom of the tubes. As judged from repeated examinations, the oil phase contained neither cells nor radioactivity in excess of the background, which indicates that complete partition of incubation fluid and lymphocytes took place. The bottom of the tubes, containing the cell pellet and a minor portion of the oil phase, was cut off and transferred to scintillation vials. The cells were then lysed in 1 ml distilled water and subsequently 5 ml scintillation fluid was added. In a number of experiments, cells were incubated with [^{14}C]-sucrose to determine trapping of incubation medium. Tritium activity was measured on an Isocap 300 scintillation counter (Nuclear, Chicago) with appropriate corrections for background activity and using the method of external channel ratio for quench correction. Counting errors less than 5% were achieved by adequate counting times.

In order to ensure optimal binding conditions during incubation, the cells were resuspended every 30 min. In addition, each sampling of cells was preceded by careful resuspension of the incubation media. In ten experiments, the cell counts in samples obtained every 30 min during 4 h incubation varied less than 6%. In all experiments, cell concentration was kept within a range of $1.5\text{--}2.0 \times 10^6$ cells/ml incubation fluid.

Data analysis

The number of ouabain molecules bound per cell was calculated from the cell count, the cellular activity uptake, the specific activity of the tracer and Avogadro's constant. The difference in activity uptake between paired vials containing tracer (total binding) and tracer + 10^{-3} M ouabain (non-specific binding) respectively equals specific receptor binding. In all experiments, measurements of both total and non-specific binding were performed in duplicate.

The amount of incubation medium, which was trapped between the cells during sampling was calculated from the [^{14}C]-sucrose activity of the cell sample and the incubation medium.

[^3H]-ouabain (O) is assumed to bind to the Na^+/K^+ ATPase receptor (R) according to the Mass Law equation

where OR represents the ouabain-receptor complex, while k_{+1} and k_{-1} denote the association and dissociation

tion rate constants respectively. In equilibration binding studies, Scatchard plots were used for estimation of the total binding capacity and the dissociation constant (K_D). According to Weiland *et al.* (1981) the binding process obeys second order kinetics given by the equation:

$$\ln \frac{B_e \times (C - (B_t \times B_e / B_{\max}))}{C \times (B_e - B_t)} = k_{+1} \times t \left(\frac{C \times B_{\max}}{B_e} - B_e \right)$$

where C is the total concentration of the tracer, $B_{\max}$ the total binding capacity, B_e the amount of drug-receptor complex at equilibrium, and B_t the amount of drug-receptor complex at time t .

However, at [³H]-ouabain concentration higher than 3×10^{-8} M, less than 0.1% of the drug is receptor bound, which simplifies the association process to (Weiland *et al.*, 1981):

$$\ln \frac{B_e}{B_e - B_t} = k_{+1} \times t \frac{B_{\max} \times C}{B_e}$$

The slope of a plot of $\ln B_e / B_e - B_t$ vs t equals $k_{+1} \times B_{\max} \times C / B_e$ which was used for calculation of the association rate constant.

The release of [³H]-ouabain from the drug-receptor complex obeys first-order kinetics given by the equation

$$B_t = B_e \times e^{-k_{-1} \times t}$$

During equilibration conditions, addition of excess unlabelled ouabain will abolish the association of labelled drug and cause a monoexponential decline of specific [³H]-ouabain binding. The dissociation rate constant was calculated from a semilogarithmic plot of these data vs time.

Results

The equilibrium binding of [³H]-ouabain to intact human lymphocytes as a function of the free [³H]-ouabain concentration is illustrated in Figure 1. The total activity uptake is composed of a large saturable component representing specific receptor binding and a minor non-saturable component representing non-specific binding. At [³H]-ouabain concentrations below 5×10^{-8} M, non-specific binding constituted less than 2% of total binding. However, with increasing tracer concentration, this component showed a steady, linear increase and at [³H]-concentration $\sim 3.5 \times 10^{-7}$ M, more than 25% of the drug was bound non-specifically.

Specific binding

It appears from Figure 1, that half maximum binding occurred at a free [³H]-ouabain concentration of

about 0.8×10^{-8} M (which equals K_D), while the usual therapeutic drug concentration was associated with a fractional receptor occupancy of less than 25%. A free drug concentration of 3.5×10^{-7} M, (which was the highest concentration used in equilibrium experiments) caused almost complete saturation of receptors, which enabled us to estimate the total binding capacity in a simple and direct manner.

The rate of specific [³H]-ouabain binding was markedly dependent on the free drug concentration as demonstrated in Figure 2. At 4×10^{-7} M, the uptake occurred rapidly, reaching equilibrium within 10 min, while 10^{-9} M caused a comparatively slow binding with at least 3 h elapsing before steady state was established.

In order to assess the incubation time necessary to accomplish equilibrium at low tracer concentration, cells were incubated in 1.5×10^{-9} M [³H]-ouabain for 5 h. As shown in Figure 3 an apparent steady state was established after about 4 h, but further incubation was associated with a slight reduction in [³H]-ouabain binding. The latter finding, which possibly represents a decrease in cell viability, was also observed during prolonged incubation with 3.5×10^{-7} M [³H]-ouabain.

Accordingly, cells were incubated up to 4 h at lower ligand concentrations during equilibrium experiments. In each vial, the presence of steady state was ensured from repeated analysis of the binding of the drug, and the values used for Scatchard analysis were the means of at least two equilibrium measurements.

A typical Scatchard plot is shown in Figure 4. The plot is strictly linear indicating the presence of a single class of receptors, and this finding was consistent in all subjects. The ordinate intercept of the regression line equals the maximum binding capacity, while the slope represents the dissociation constant (K_D).

The association rate constant (k_{+1}) was calculated from uptake curves using $3-4 \times 10^{-8}$ M tracer concentration as demonstrated in Figure 5. As anticipated from the linearity of the Scatchard analysis, a plot of

$$\ln \frac{B_e}{B_e - B_t}$$

vs time also yielded a straight line in all subjects. The slope of the regression line

$$\left(\text{which equals } k_{+1} \times \frac{C \times B_{\max}}{B_e} \right)$$

enabled calculations of k_{+1} , in that the maximum binding capacity was measured in simultaneously performed equilibrium experiments.

While the specific binding of [³H]-ouabain occurred rapidly, the dissociation of the drug-receptor complex was a comparatively slow process. Figure 6 demonstrates the time course of release of [³H]-ouabain after addition of excess unlabelled drug at equilibrium. The amount of specifically bound [³H]-

Figure 1 Total [³H]-ouabain uptake (●), specific [³H]-ouabain binding (○) and non-specific [³H]-ouabain binding (■) in human lymphocytes related to the free [³H]-ouabain concentration. Mean ± s.d. from six experiments.

Figure 2 The time course of the specific [³H]-ouabain binding at different free tracer concentration in a typical subject. Each point represents the mean of two measurements.

ouabain is expressed as a percentage of the value measured before cold ouabain is added. The binding of the radioligand showed a typical monoexponential decay.

Non-specific binding

It appears from Figure 1, that the amount of non-specific [³H]-ouabain binding increased in a linear fashion with the free tracer concentration. To evaluate whether this binding was due to intracellular accumulation of drug or simply represented trapping of incubation medium, parallel measurements of [¹⁴C]-sucrose and non-specific [³H]-ouabain binding were performed (Figure 7). The space available for [¹⁴C]-sucrose, which represents the extracellular trapped volume, amounted to about 60 nl/10⁶ cell and remained constant during 2 h incubation. This value agrees within experimental error with the [³H]-ouabain space (when receptors are saturated with unlabelled drug), indicating that non-specific [³H]-ouabain uptake within the first 2 h of incubation can be explained simply by extracellular trapping of the drug. In more prolonged experiments, however, the non-specific [³H]-ouabain uptake showed a slight tendency to increase, which possibly reflects additional intracellular accumulation. In four experiments, parallel incubation of cells in [¹⁴C]-sucrose and ³H₂O revealed a ³H₂O-space of 395 ± 62 nl/10⁶

Figure 3 The time course of the specific [³H]-ouabain binding from four subjects during 5 h incubation at 1.5×10^{-9} M radioligand concentration. Each point is the mean of two measurements.

cells and a mean lymphocytic volume (³H₂O-space less [¹⁴C]-sucrose-space) of 314 ± 58 nl/ 10^6 cells. These results are in good accord with values from the literature (Wintrobe, 1974) in that our harvests probably comprise mainly smaller lymphocytes.

[³H]-ouabain binding in normal individuals

Thirty-two healthy hospital employees, aged 22–35 years were examined. All were medicine-free and without identifiable disease. Blood pressure was normal and none reported an hereditary disposition for hypertension. The results of the binding studies are listed in Table 1. In thirteen individuals, lymphocytes (from which monocytes were removed through defibrination) had a mean binding capacity of 42070 ± 6851 molecules/cell and a mean apparent dissociation constant (K_D) of $0.675 \pm 0.207 \times 10^{-8}$ M as determined from Scatchard analysis. Direct estimation of the binding capacity from equilibrium binding at 3.5×10^{-7} M [³H]-ouabain gave essentially identical result

Figure 4 Equilibrium binding data presented as a Scatchard plot. Ordinate intercept indicates the maximum binding capacity and the slope represents the dissociation constant (K_D).

(42482 ± 8369 molecules/cell). As shown in Figure 8, the two parameters were strongly intercorrelated ($r = 0.97$, $n = 13$, $P < 0.001$) and the regression line did not differ significantly from the line $y = x$. Accordingly, this direct method which was performed in further twelve individuals seems to determine the binding capacity with sufficient accuracy. The pooled data showed a total binding capacity of 43154 ± 8037 molecules/cell ($n = 25$).

In three subjects, direct determination of the [³H]-ouabain binding capacity was performed in duplicate in five consecutive days. These data revealed total coefficients of variation between 7–9% and a within coefficient of variation (normalized data) of 7%.

In six experiments, the mean association and dissociation rate constants were $3.079 \pm 0.340 \times 10^4$ /M/s and $1.578 \pm 0.499 \times 10^{-4}$ /s, respectively. The derived dissociation constant (k_{-1}/k_{+1}) equals 0.512×10^{-8} M, which agrees within experimental error with the value calculated from Scatchard plots.

When cells were isolated without the use of defibrination a substantial contamination with monocytes resulted. In Figure 9, the [³H]-ouabain binding to these mixtures of mononucleated cells is compared to pure lymphocyte preparations from the same six individuals. Mononucleated cells had a markedly higher binding capacity than that of lymphocytes, but the dissociation constants were almost identical (Table 1). In a total of nine subjects, mononucleated cells had a mean binding capacity of 75474 ± 6764 molecules/cell (Figure 10).

Discussion

The use of Scatchard analysis for evaluation of receptor binding kinetics is based on the assumption that equilibrium is reached at each radioligand concentration. Our findings indicate that this condition was fulfilled after about 2 h incubation at radioligand concentration above 10^{-8} M, whereas lower ligand concentrations required about 4 h of incubation before an apparent steady-state was reached (Figure 3). The duration of incubation might have been extended considerably further, since the relative amount of non-specific [³H]-ouabain uptake remained low. However, between 4–5 h there was a slight, but consistent, reduction of radioligand binding which may be attributed to a change in cell viability. Accordingly we consider 4 h as the incubation time limit when lymphocytes are used.

The present results indicate that the normal lymphocyte possesses a finite number of [³H]-ouabain receptors with a high affinity for this drug. The strict linearity of the plot of

$$\ln \frac{B_e}{B_e - B_t} \text{ vs time}$$

Figure 5 (a) Time course of the specific $[^3\text{H}]$ -ouabain binding at 4.0×10^{-8} M free tracer. Each point is the mean of two measurements.

(b) Plot of $\ln B_e/(B_e - B_t)$ vs time using experimental data from a (B_e : equilibrium binding, B_t : binding at time t).

Figure 6 The rate of dissociation of the $[^3\text{H}]$ -ouabain receptor complex. Cells were allowed to equilibrate at 4.0×10^{-8} M tracer concentration; 10^{-3} M unlabelled ouabain was then added and the fractional receptor occupancy was measured with the specified time intervals. Each point represents the mean of two measurements.

Figure 7 Space available for [³H]-ouabain (in the presence of 10⁻³ M unlabelled ouabain) (○) and [¹⁴C]-sucrose (●). The values were calculated from the activity of the supernatant, the cell activity uptake and the specific activity of the isotope. Mean ± s.d. of five experiments.

Figure 8 Relationship between [³H]-ouabain binding capacity of lymphocytes calculated by Scatchard analysis and measured directly at 3.5 × 10⁻⁷ M tracer concentration. Regression line, $y = 1.155x - 5.777$ ($r = 0.970$, $n = 13$, $P < 0.001$) was not significantly different from $y = x$ (95% confidence limits for slope: 1.324, 0.986 and ordinate intercept: 1453, -13007).

(Figure 5) and the Scatchard plot suggests the presence of a single class of receptors without signs of negative or positive cooperativity.

In equilibrium experiments we found a mean dissociation constant (K_D) of 0.675×10^{-8} M derived from Scatchard plots. The possibility exists that this estimate may slightly overestimate the actual value, since limited cell viability may have prevented a true steady state to be established at low radioligand concentration. However, this problem seems not to affect determination of the maximum binding capacity, since we found good accordance between B_{max}

values measured directly and calculated from Scatchard plots (Figure 8). In uptake and release studies (Figure 5 and Figure 6), the use of rather high radioligand concentrations ensured a true equilibrium to be accomplished within about 2 h. The mean dissociation constant (k_{-1}/k_{+1}) calculated from these experiments was 0.512×10^{-8} M, which is only slightly less the value derived from Scatchard analysis. Thus our equilibrium studies seem to provide a reasonably good estimate of [³H]-ouabain affinity.

Our results indicate that non-specific [³H]-ouabain binding predominantly consists of extracellular trapping of the drug and that only small amounts of [³H]-ouabain enter the cells, when the receptors are occupied by excess unlabelled drug. This finding together with the rapidity of specific [³H]-ouabain

Table 1 Binding capacity and kinetic constants for the specific [³H]-ouabain uptake in lymphocytes and mononucleated cells. Mean ± s.d. values.

Lymphocytes	
Binding capacity ($n = 25$)	43154 ± 8037 molecules/cell
Association rate constant, k_{+1} ($n = 6$)	$3.079 \pm 0.340 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$
Dissociation rate constant, k_{-1} ($n = 6$)	$1.578 \pm 0.499 \times 10^{-4} \text{ s}^{-1}$
Dissociation constant, K_D	
$\frac{k_{-1}}{k_{+1}}$ ($n = 6$)	$0.512 \pm 10^{-8} \text{ M}$
Scatchard ($n = 13$)	$0.675 \pm 0.207 \times 10^{-8} \text{ M}$
Mononuclear cells	
Binding capacity ($n = 9$)	75474 ± 6764 molecules/cell
Dissociation constant, K_D ($n = 6$)	$0.631 \pm 0.123 \times 10^{-8} \text{ M}$

Figure 9 Equilibrium binding of [^3H]-ouabain to mixtures of mononucleated cells (●) and pure lymphocyte preparations (○) obtained from the same six individuals. Dotted lines indicate total binding capacity.

uptake support the concept of the receptor being located at the outer surface of the cell membrane. Additional specific binding to intracellular structures cannot be ruled out for certain, but seems quantitatively infinitesimal.

The number of [^3H]-ouabain binding sites per lymphocyte in the present investigation is essentially identical to the results of Clausen *et al.* (1980), who used the same experimental procedure. Lymphocytes seem to possess on average 200-fold binding sites per cell compared to erythrocytes (Erdmann *et al.*, 1975; Ford *et al.*, 1979; Kaji *et al.*, 1981). Since the cell surface of the erythrocyte is only slightly less that of the lymphocyte, the latter has clearly a much higher receptor density. However, the dissociation constants of erythrocyte receptors obtained by Gardner *et al.* (1972) ($0.625 \times 10^{-8} \text{ M}$) and Erdmann *et al.* (1975) ($0.3 \times 10^{-8} \text{ M}$) were within the same order of magnitude as our results, indicating structural identity of the receptor. In animal studies, Baker & Willis (1972) found a similar difference in ouabain binding site densities between erythrocytes and a variety of other tissues. The erythrocyte had about 1 binding site/ μm^2 cell surface (similar in man) compared to more than 500 sites/ μm^2 in other tissues, and a corresponding difference in $\text{Na}^+\text{K}^+\text{ATPase}$ activity was observed.

In a mixture of mononucleated cells, the binding sites were almost double compared with a pure lymphocyte preparation. Accordingly, monocytes seem to possess substantially more binding sites than lymphocytes, possibly due to their larger size. This finding emphasizes the importance of well-defined cell populations when the number of pumps between

Figure 10 Total [^3H]-ouabain binding capacity of lymphocytes ($n = 25$) and mononucleated cells ($n = 9$).

individuals is compared or when individuals are examined over a period of time.

Our results indicate that direct determination of [^3H]-ouabain binding can be performed with sufficient precision and provides a reliable estimation of the actual number of binding sites extrapolated from Scatchard plots. This method demands only about 30 ml whole blood compared to 120 ml for Scatchard analysis, and seems preferable in a clinical setting, when a mere number of pump sites is required.

In conclusion, the lymphocytes are an accessible cell model for investigation of Na^+/K^+ pump function in man. A mixture of mononucleated cells seems less appropriate since the monocyte fraction may fluctuate, possibly leading to substantial variation in pump sites. Further studies must clarify whether the lymphocyte is superior to the erythrocyte as a peripheral marker of pump function in tissues.

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