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Effects of Cations on Ouabain Binding by Intact Human Erythrocytes

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Summary. The present studies demonstrate that extracellular cations alter ouabain binding by intact human erythrocytes and that this alteration reflects a change in the apparent affinity but not the capacity for ouabain binding. Monovalent cations exert their effect at a single site (the "monovalent cation site") and each monovalent cation can inhibit competitively the effect of other monovalent cations. Sodium, lithium, and cesium increase while potassium and rubidium decrease the apparent affinity with which ouabain is bound. Divalent cations exert their effect at a single site (the "divalent cation site") which is functionally distinct from the site at which monovalent cations act and show mutual competitive inhibition. Each divalent cation studied increases the apparent affinity with which oubain binds to the erythrocyte membrane. Magnesium and calcium, but not barium, can also alter the effect of monovalent cations on ouabain binding. To interpret our findings we have proposed that the erythrocyte membrane has a "receptor complex" composed of a monovalent cation site, a divalent cation site and a glycoside-binding site. The number and type of cations occupying the cation sites determine the affinity of the glycoside-binding site for ouabain.

Both cation transport by intact cells and Na,K-dependent ATPase activity in broken cell preparations are inhibited by low concentrations of cardioactive glycosides [14–16, 23, 29, 33–36] and Kyte, using a kinetic titration technique, has found that glycosides bind to a purified, soluble ATPase preparation which is composed of two polypeptides [25–27]. One approach to understanding further the mechanism by which glycosides alter membrane function is to focus directly on their interaction with the cell membrane. Glycoside binding to intact cells, to resealed ghosts and to broken cell membrane preparations has been found to depend on the cation composition of the incubation solution [1–4, 8–10, 17, 18, 26, 28, 36]; however, the effects of extracellular cations have not been examined syste-

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matically. Furthermore, no clear distinction has been made between effects of cations on glycoside binding and effects on glycoside-sensitive cation transport.

The effects of extracellular sodium and potassium on ouabain binding to intact human erythrocytes result from changes in the affinity but not the capacity for glycoside binding [10]. In the present experiments we have examined systematically the effects of monovalent and divalent cations on ouabain binding by intact human erythrocytes. These studies should serve both to clarify the functional characteristics of the mechanism by which ouabain is bound to the cell membrane and to establish a basis for distinguishing effects of extracellular cations on glycoside-sensitive cation transport from their effects on ouabain binding.

Materials and Methods

^{3}H-Ouabain (Lot No. 184–263, specific activity 11.7 C/mmole) was obtained from New England Nuclear Corp. ^{42}K (specific activity 120–150 C/mmole) was obtained as the chloride salt from International Chemical and Nuclear Corporation. All metal cations were obtained as the chloride from Fisher Scientific Co., choline chloride was from Eastman Kodak Co. and Tris was from Schwarz/Mann.

Erythrocytes obtained from eight normal male and female human volunteers (26 to 31 years of age) were washed three times in isosmotic choline chloride (pH 7.4). Ouabain binding was determined as described previously [10]. Erythrocytes were added to incubation solutions (prewarmed to 37 °C) containing ^{3}H-ouabain. The hematocrit of the incubation mixture was 5 to 10%. Triplicate 100-μliter samples were taken after at least 3 hr of incubation, placed in polyethylene micro test tubes (Beckman Instruments Inc.) and washed 5 times with 300 μliters of isosmotic choline chloride by alternate centrifugation at 10,000 × g for 15 sec and resuspension. Appropriate control experiments showed that incubation for 3 hr was sufficient for the binding reaction to reach a steady state. Centrifugation was performed using a Microfuge® (Beckman Instruments Inc.). After the final wash, each sample was treated with 100 μliters of 10% perchloric acid, agitated and centrifuged for 30 sec. The tube containing the sample was inverted and placed in a vial containing 20 ml of liquid scintillation solution. When the vial was shaken, the supernatant passed from the sample tube into the scintillant and the precipitate remained in the tip of the sample tube.

At some time during the incubation, triplicate 100-μliter samples of the incubation mixture were added to 100 μliters of 10% perchloric acid, agitated, centrifuged, inverted and placed in a vial containing liquid scintillation solution. The volume of cells counted was calculated from the hemoglobin concentration of the incubation mixture and the hemoglobin concentration and hematocrit determined on a separate tube containing the incubation solution and erythrocytes at a hematocrit of approximately 25%. The hematocrit was measured using a Drummond microhematocrit centrifuge and hemoglobin concentration was measured using the cyanmethemoglobin method [6].

The standard incubation solution had the following composition (mM): choline chloride, 150; Tris-HCl (pH 7.4), 10; glucose, 11.1. The chloride salts of the various cations studied were added to the incubation medium and an osmotically equivalent amount of choline chloride was removed.

The amount of ouabain bound was calculated from the counts per milliliter of cells and the specific activity of ouabain in the incubation medium. The concentration of ouabain in the ^{3}H-ouabain supplied by the manufacturer was verified by measuring the binding of radioactivity in the presence of constant ^{3}H-ouabain and varying concentrations of nonradioactive ouabain [10]. Binding of radioactive impurities or entrapment of ^{3}H-ouabain was determined from the number of counts bound in the presence of 10,000-fold molar excess of nonradioactive ouabain.

Liquid scintillation counting was performed using 20 ml of a solution composed of 15 parts toluene (J. T. Baker Chemical Co.), 5 parts Triton X-100® (New England Nuclear Corp.) and 1 part Liquifluor® (New England Nuclear Corp.). The observed counts were usually such that their standard deviation was less than 2%. Variation in quenching was monitored by using the ratio of counts in two channels produced by an automatic external standard; however, since in all cases the maximum range was less than 1.8%, no quench correction was made.

The relation between ouabain binding and cation transport was explored by measuring potassium influx on cells which had been incubated with or without ^{3}H-ouabain. At appropriate times triplicate samples were taken for determination of ouabain binding and the remaining cells were washed four times with at least 30 volumes of cold (4 °C) isosmotic choline chloride. To determine potassium influx these washed cells were added to incubation solutions (37 °C) containing ^{42}K. The hematocrit was approximately 4%. After mixing thoroughly, duplicate 100-µliter samples were placed in polyethylene micro test tubes and washed four times with 300 µliters of cold, isosmotic sodium chloride. After the final wash each sample was treated with 100 µliters of 10% perchloric acid, agitated, centrifuged, inverted and placed in 20 ml of liquid scintillation fluid for counting. At some time during the incubation, triplicate 100-µliter samples of the incubation mixture (i.e. cells plus medium) were added to a micro test tube containing 100 µliters of 10% perchloric acid, agitated, centrifuged, inverted and placed in liquid scintillation solution. The volume of cells counted was calculated from the hemoglobin concentration in the incubation mixture and the previously measured hemoglobin content per volume of cells. The incubation solution used to determine potassium influx had the following composition (mM): NaCl, 150; KCl, 15; Tris-HCl (pH 7.4), 10; glucose, 11.1. The sodium and potassium concentrations of the incubation solutions were also determined at the end of the incubation period. Since the uptake of ^{42}K was observed to be constant during a 45-min incubation period, potassium influx was calculated from samples taken at zero and 40 min. Potassium influx was calculated using the method described by Sachs and Welt [31] and the average of the potassium concentrations in the incubation solutions at zero and 40 min. All counts were corrected for decay. Sodium and potassium concentrations were measured with an Instrumentation Laboratories Model 143 flame photometer.

Results

In a previous study [10] of the effects of extracellular sodium and potassium on ouabain binding to intact human erythrocytes we found that ouabain binding could be described by the equation

$$A_B = B_{\max}\left(\frac{A}{A+K_B}\right)$$

where A_B is the amount of ouabain bound, $B_{\max}$ is the maximum amount of ouabain which can be bound, A is the ouabain concentration in the incuba-

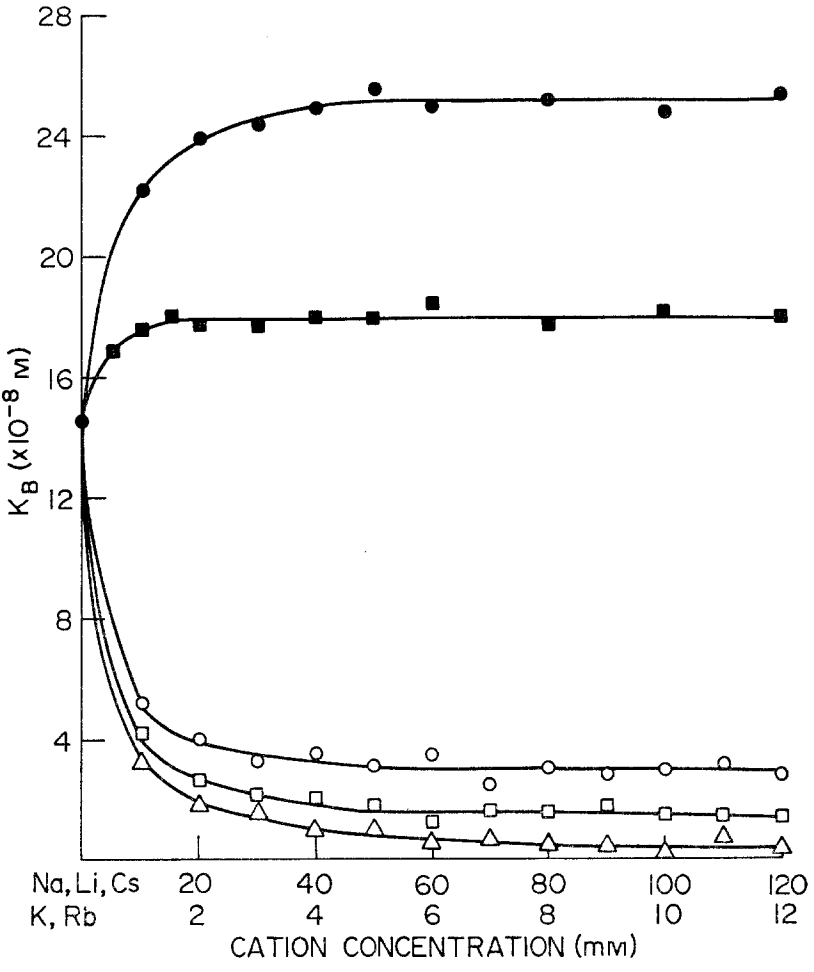

Fig. 1. Value of K_B for ouabain binding at different concentrations of potassium (closed circles), rubidium (closed boxes), cesium (open circles), lithium (open boxes) or sodium (open triangles). Each point represents the mean of 4 experiments

tion solution and K_B is the ouabain concentration at which binding is half-maximal. Both sodium and potassium altered K_B but had no effect on B_{max} [10].

In the present experiments at different concentrations of lithium, cesium or rubidium, double-reciprocal plots of ouabain bound versus ouabain concentration gave straight lines with a common nonzero intercept. That is, these cations, like sodium and potassium, altered K_B but had no significant effect on B_{max}. To explore further the effect of these cations, B_{max} was determined from triplicate samples taken from each of three incubation tubes containing sufficiently high concentrations of ^{3}H-ouabain

Table 1. Lack of effect of choline replacement or hydrogen ion concentration on ouabain binding

Composition of incubation solution	Ouabain bound (pmoles/ml cells)	
	Ouabain (7.17×10^{-7} M)	Ouabain (1.23×10^{-8} M)
Choline, 150 mM	18.50 ± 1.21	1.70 ± 0.12
Choline, 100 mM; sucrose, 100 mM	18.34 ± 1.36	1.68 ± 0.06
Choline, 50 mM; sucrose, 200 mM	17.91 ± 1.46	1.61 ± 0.18
Sucrose, 300 mM	18.88 ± 1.39	1.82 ± 0.15
Choline, 150 mM, pH = 5.0	18.71 ± 1.17	1.82 ± 0.09
Choline, 150 mM, pH = 6.0	18.40 ± 1.33	1.86 ± 0.09
Choline, 150 mM, pH = 7.0	19.08 ± 0.25	1.68 ± 0.16
Choline, 150 mM, pH = 8.0	17.65 ± 1.38	1.75 ± 0.09

Each value represents mean ± SD of 4 experiments. Unless otherwise specified the pH of all incubation media was 7.4. Each incubation solution also contained Tris-HCl (pH 7.4), 10 mM and glucose, 11.1 mM.

and cations to produce maximal binding[1]. Then by measuring the amount of ouabain bound (A_B) in a particular incubation solution containing a known concentration of ^{3}H-ouabain (A) the value of K_B was calculated.

As the extracellular concentration of lithium or cesium was increased there was a progressive decrease in K_B (Fig. 1), and at a given concentration the relative effect of these cations on K_B was Na > Li > Cs. Conversely, the relative ability of each cation to exert its maximal effect on K_B was Cs > Li > Na. As the extracellular concentration of rubidium increased there was a progressive increase in K_B (Fig. 1). At a given concentration potassium produced a greater increase in K_B than did rubidium; however, the relative ability of each cation to exert its maximal effect on K_B was Rb > K.

The data in Table 1 show that ouabain binding was not altered significantly when choline chloride was replaced by an isosmotically equivalent

1 Our value for B_{max} indicates that there are approximately 1,200 ouabain-binding sites per cell. This value is significantly higher than values reported by others using different techniques [3, 17, 18], and as we have pointed out [10], part of this discrepancy is probably attributable to differences in the method for extracting bound radioactivity from the cells, in the composition of the liquid scintillation solution and in the procedure used to correct for variable counting efficiency. Another potential source of this discrepancy may be that previous studies of ouabain binding to human erythrocytes were performed using different lots of ^{3}H-ouabain (at a specific activity 3 to 25 times lower) than were used for the present experiments [3, 17, 18]. Dunham and Hoffman [8] have indicated that the wide range of values obtained in different laboratories for ouabain binding to sheep erythrocytes might be attributable to differences which they found between two preparations of ^{3}H-ouabain from the same commercial supplier.

amount of sucrose or when the hydrogen ion concentration in the incubation medium was varied from 10^{-5} to 10^{-8} M.

Adding calcium, barium or magnesium to the incubation solution increased both the rate of ouabain binding and the amount bound at the steady state. At different concentrations of each of these divalent cations double-reciprocal plots of ouabain bound versus ouabain concentration resulted in straight lines with a common nonzero intercept. As the extracellular concentration of calcium, barium or magnesium was increased, there was a progressive decrease in K_B (Fig. 2). At each concentration studied, the relative effect of these cations on K_B was Mg > Ba > Ca. Conversely, the relative ability of a particular divalent cation to exert its maximal effect on K_B was Ca > Ba > Mg.

To account for the effects of extracellular sodium and potassium on ouabain binding by human erythrocytes we have postulated previously [10] that the erythrocyte membrane contains a finite number of receptors each composed of a glycoside-binding site and a cation site and that each site can associate reversibly with one glycoside molecule and one cation, respectively.

To test this latter assumption of a 1:1 correspondence between the number of glycoside-binding sites affected and the number of cation sites occupied we compared the observed effects of cesium on K_B with values expected if the ratio of glycoside sites affected/cation sites occupied is not 1:1. Using equations derived previously[2] [10] values of K_B predicted at each of the six cesium concentrations studied, assuming that the alteration of K_B was related to $[Cs]^x$ where $x = 2$, 1.5, 0.667 and 0.50, were significantly different from observed values (Fig. 3). A value of x of 0.95 to 1.05 was required for each of the predicted values to be within one standard deviation of the observed values. Similar results were obtained when the same experiment was performed with rubidium or with magnesium.

Using the results given in Figs. 1 and 2 we have calculated values for the apparent dissociation constants for the interaction between ouabain and

2 The equations used to describe the interaction between ouabain and the erythrocyte membrane, as well as how this interaction depends on the cation composition of the incubation solution, were derived using the same conceptual approach and assumptions set forth previously [10]. In particular, we have assumed that the reactions between ouabain and the glycoside-binding site and between cations and the cation-binding sites have reached a steady state and that at the steady state the concentrations of cations, ouabain, free binding sites and occupied binding sites can be described by a series of "dissociation constants". From these relations an equation can be written which gives the amount of ouabain bound as a function of the maximal ouabain binding capacity, the ouabain concentration and the types and concentrations of cations in the incubation solution.

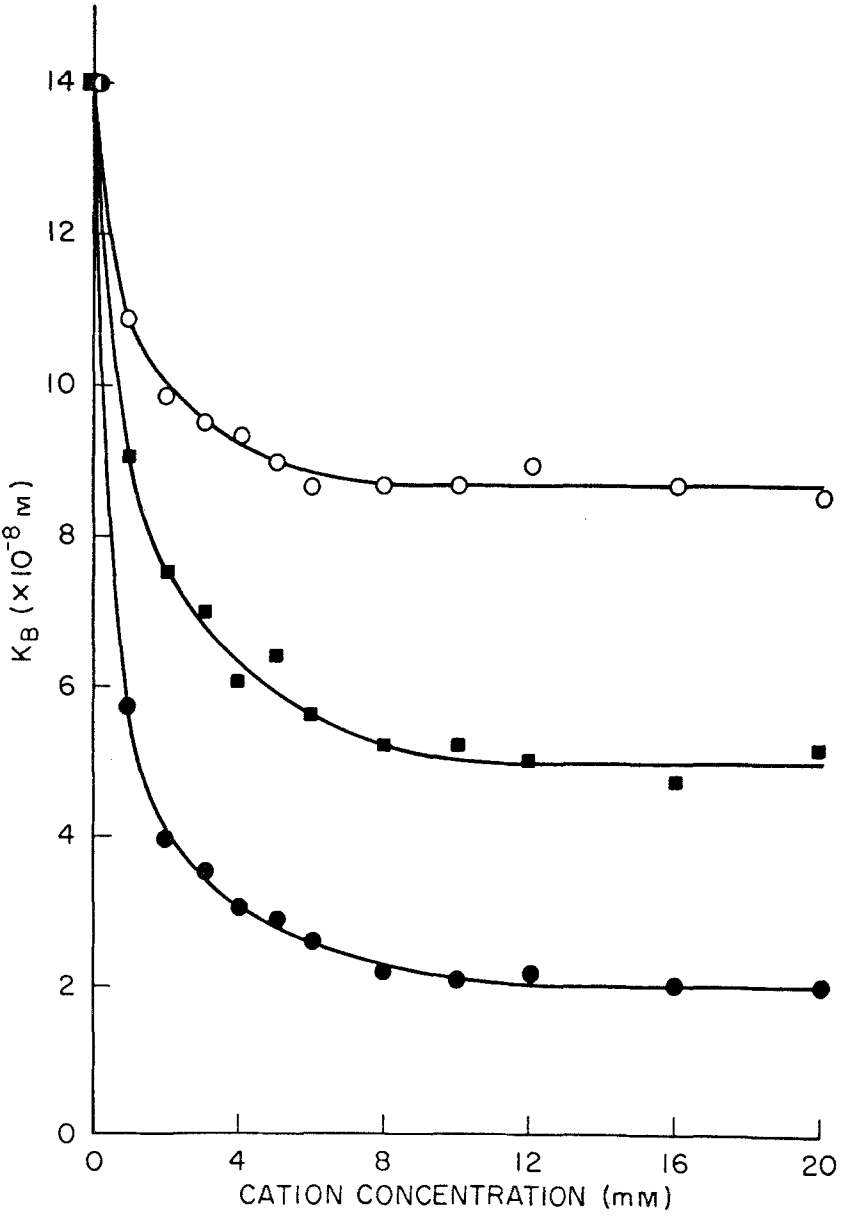

Fig. 2. Value of K_B for ouabain binding at different concentrations of calcium (open circles), barium (boxes) or magnesium (closed circles). Each point represents the mean of 4 experiments

the glycoside-binding site and for the interaction between cations and the cation site[2] (Table 2). We then compared values for ouabain binding measured in the presence of various combinations of monovalent cations with those predicted from the values given in Table 2. This was also done

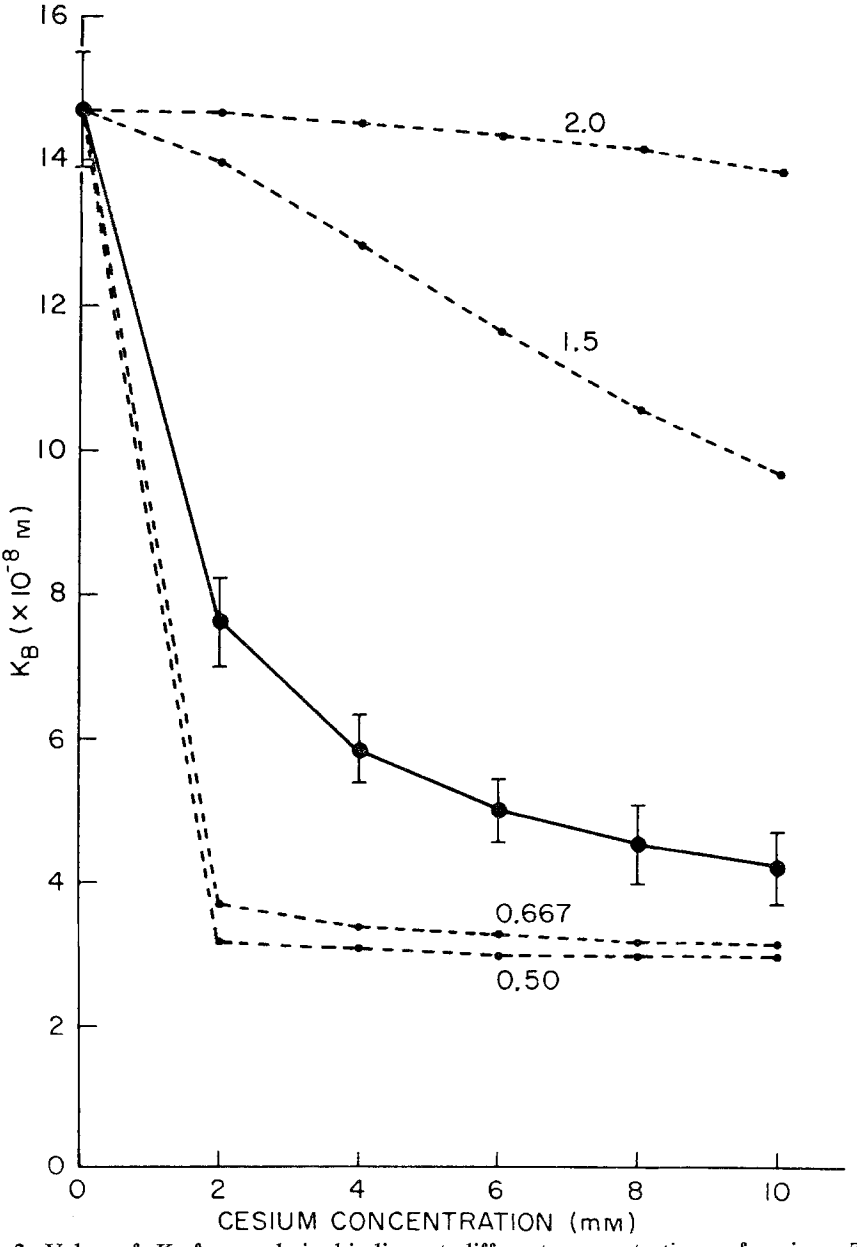

Fig. 3. Value of K_B for ouabain binding at different concentrations of cesium. The values connected by a solid line were obtained as described in the text. Each point represents the mean of 5 experiments and the vertical lines represent ± 1 SD. The predicted values connected by dashed lines were calculated using the values in Table 2 and assuming that K_B was a function of $[Cs]^x$ where $x = 2.0$, 1.5, 0.667 and 0.50

using various combinations of divalent cations. As illustrated by the data in Table 3, there was good agreement between predicted and observed values. However, when ouabain binding was measured in the presence of

Table 2. Dissociation constants which describe the interaction of ouabain and cations with the erythrocyte membrane [a]

Cation	Dissociation constant (M)	
	Cation site	Glycoside site
Na	$918\ (\pm 217) \times 10^{-3}$	5.0×10^{-10} [b]
Li	$105\ (\pm 9.1) \times 10^{-3}$	$9.7\ (\pm 2.4) \times 10^{-9}$
Cs	$7.2\ (\pm 2.4) \times 10^{-3}$	$2.8\ (\pm 0.6) \times 10^{-8}$
K	$0.28\ (\pm 0.17) \times 10^{-3}$	$2.55\ (\pm 0.48) \times 10^{-7}$
Rb	$0.13\ (\pm 0.08) \times 10^{-3}$	$1.81 \pm 0.24) \times 10^{-7}$
Ca	$1.2\ (\pm 0.4) \times 10^{-3}$	$8.3\ (\pm 0.8) \times 10^{-8}$
Ba	$2.7\ (\pm 0.3) \times 10^{-3}$	$4.5\ (\pm 0.6) \times 10^{-8}$
Mg	$4.0\ (\pm 1.2) \times 10^{-3}$	$1.7\ (\pm 0.2) \times 10^{-8}$

[a] For each individual experiment the values for the apparent dissociation constants were calculated using a nonlinear least-squares technique [7] and the equations derived previously[2] [10]. The results given are the means (± 1 SD) of the experiments reported in Figs. 1 and 2. The dissociation constants for the cation site represent the cation concentration at which 50% of the cation sites are occupied by a particular cation. The dissociation constants for the glycoside site represent the ouabain concentration at which ouabain binding is half-maximal when the cation site is completely occupied by the indicated cation.

[b] This value is the one given in a previous paper [10]. The dissociation constant for the glycoside site in the presence of 150 mM choline was $1.47\ (\pm 0.27) \times 10^{-7}$ M. B_{max} was 22.3 (± 1.9) pmoles/ml cells.

Table 3. Comparison of observed and calculated values for ouabain binding in the presence of various combinations of cations

Composition of incubation solution (mM)	Ouabain bound (pmoles/ml cells)	
	Observed	Calculated [a]
Choline = 150	1.87 ± 0.19 [b]	1.96
Na = 75	14.90 ± 1.68	14.11
Li = 75	6.30 ± 1.30	7.54
Cs = 75	3.10 ± 0.86	3.43
K = 10	1.11 ± 0.18	1.15
Rb = 10	1.43 ± 0.08	1.55
Na = 75; Li = 75	12.60 ± 1.21	13.18
Na = 75; Cs = 75	8.02 ± 0.51	7.79
Na = 75; K = 10	2.32 ± 0.18	2.27
Na = 75; Rb = 10	2.01 ± 0.10	2.08
Mg = 10	7.63 ± 0.49	7.50
Ba = 10	4.27 ± 0.26	4.39
Ca = 10	2.81 ± 0.27	2.60
Mg = 10; Ca = 10	5.43 ± 0.31	5.47
Mg = 10; Ba = 10	5.39 ± 0.36	5.62

[a] Calculated from the values given in Table 2 using equations derived previously[2] [10].
[b] Mean of 3 experiments ± 1 SD. Each incubation solution also contained Tris HCl (pH 7.4), 10 mM; glucose, 11.1 mM and ^{3}H-ouabain, 1.5×10^{-8} M.

Table 4. Comparison of observed and calculated values for ouabain binding in the presence of various combinations of monovalent and divalent cations

Composition of incubation solution (mM)	Ouabain bound (pmoles/ml cells)	
	Observed	Calculated [a]
Na = 75; Ca = 10	16.30 ± 0.93 [b]	4.81
Na = 75; Ba = 10	15.90 + 0.87	6.07
Na = 75; Mg = 10	18.06 ± 1.32	6.40
Mg = 10; Li = 75	8.02 ± 0.42	9.33
Mg = 10; Cs = 75	6.59 ± 0.43	6.82
Mg = 10; K = 10	1.38 ± 0.08	1.83

[a] Calculated using the values given in Table 2 using equations derived previously[2] [10].
[b] Each value represents the mean of 4 experiments ±1 SD. Each incubation solution also contained Tris-HCl (pH 7.4), 10 mM; glucose, 11.1 mM and ^{3}H-ouabain, 1.5×10^{-8} M.

a monovalent cation and a divalent cation there was poor agreement between observed and predicted values (Table 4). This poor agreement suggested that the site at which monovalent cations act to alter ouabain binding is functionally distinct from the site at which divalent cations act.

To explore the possible interactions between monovalent cations and divalent cations we studied the effect of magnesium on K_B in the presence of different monovalent cations. When potassium (20 mM), rubidium (20 mM), cesium (120 mM) or lithium (120 mM) were present in the incubation solution, magnesium produced no detectable alteration in the value of K_B (Fig. 4). We then examined the effect of potassium on K_B in the presence of magnesium. In both the presence and absence of magnesium (20 mM), as the potassium concentration increased, K_B increased steadily until it reached a maximum value (Fig. 5). Furthermore, the maximum value of K_B was the same both with and without magnesium. Fig. 5 also illustrates the results of a similar experiment performed using cesium, a monovalent cation which increases the apparent affinity of ouabain binding (*see* Fig. 1). In the absence of magnesium as the cesium concentration increased, K_B decreased steadily until it reached minimum value. In the presence of 20 mM magnesium, as the cesium concentration increased, K_B increased until it reached a maximum. Furthermore, at cesium concentrations greater than approximately 25 mM, K_B was independent of the magnesium concentration and did not change with further increases in the cesium concentration. In other words, addition of low concentrations of cesium (less than approximately 25 mM) to a magnesium-free solution produced an increase in the apparent ouabain binding affinity; however, in the presence of 20 mM magnesium, adding low concentrations of cesium decreased the ouabain binding affinity.

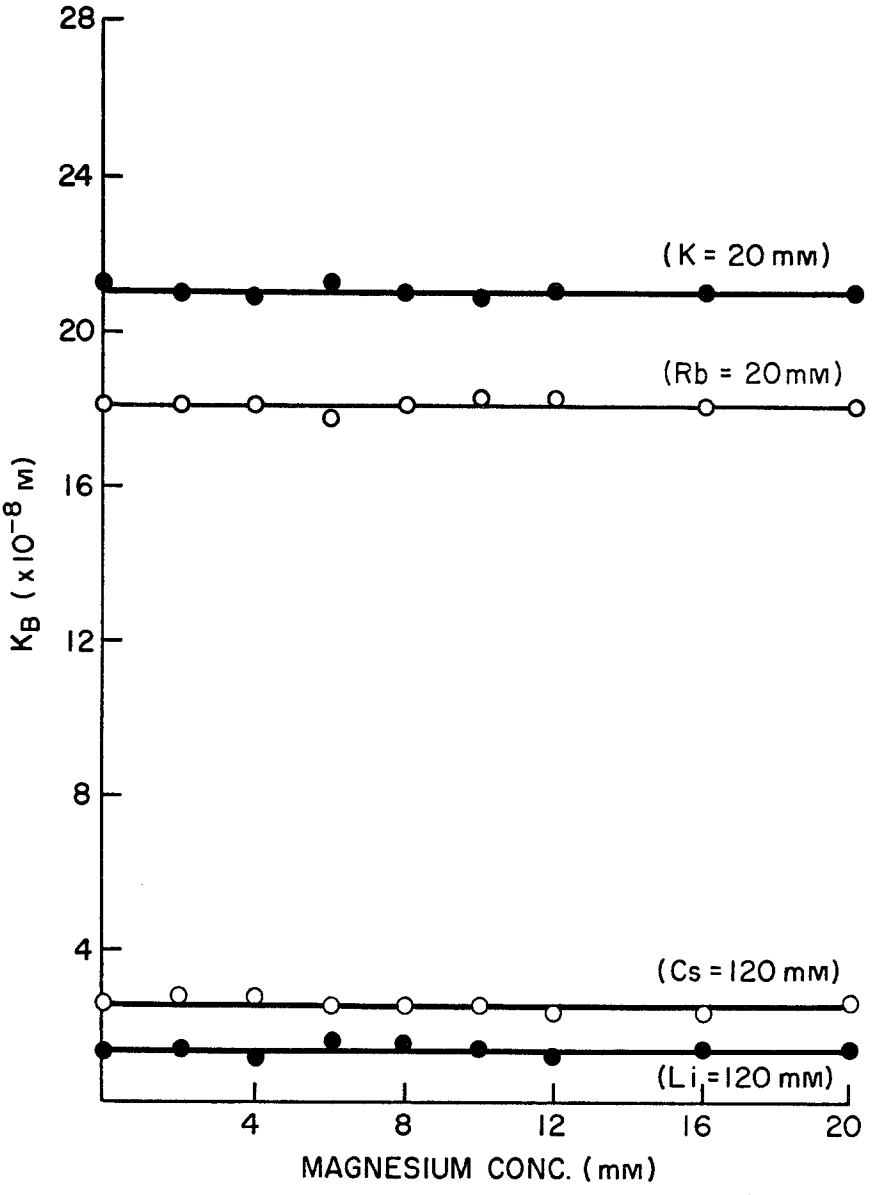

Fig. 4. Absence of an effect of magnesium on the values of K_B for ouabain binding in the presence of potassium, rubidium, cesium and lithium. Each point represents the mean of 3 experiments

To explore the effects of divalent cations on the apparent affinity of monovalent cations for their cation site, the value of K_B for ouabain binding was determined at various potassium concentrations in the presence of 20 mM magnesium. These values were then compared with values calculated using

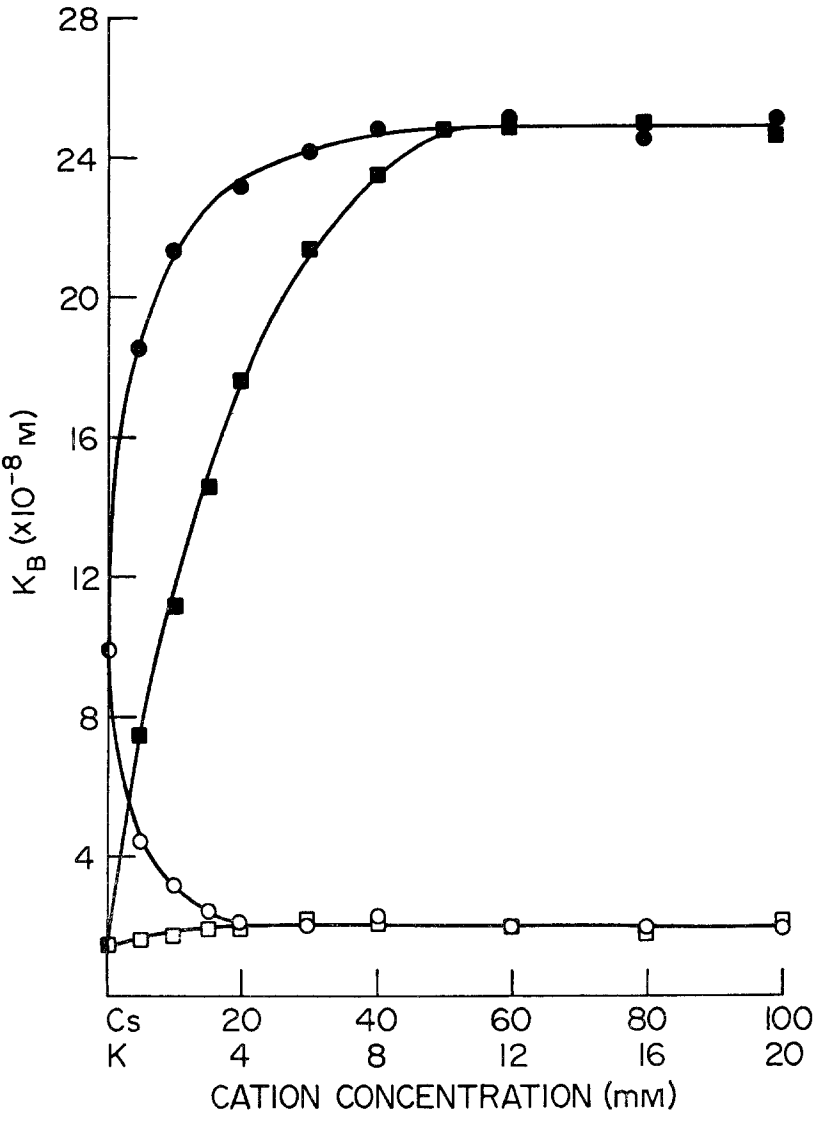

Fig. 5. Effect of potassium (closed boxes and circles) and cesium (open boxes and circles) on the value of K_B for ouabain binding in the presence (boxes) and absence (circles) of 20 mM magnesium. Each point represents the mean of 3 experiments

the dissociation constants given in Table 2 and assuming that the affinity of potassium for its cation site was not altered by magnesium. In the presence of magnesium, values of K_B were significantly greater than values calculated on the assumption that the affinity of potassium for its site was not altered by magnesium (Fig. 6). Conversely, in the presence of calcium, the values of K_B were significantly lower than those expected if the affinity

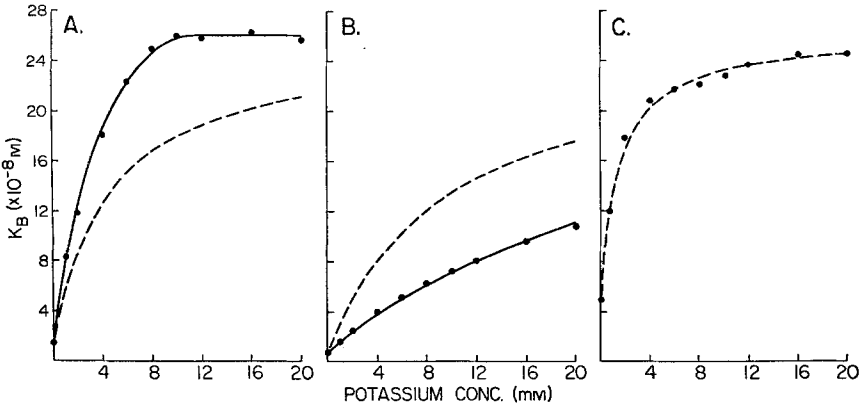

Fig. 6. Effect of potassium on the value of K_B for ouabain binding in the presence of magnesium (panel *A*), calcium (panel *B*) or barium (panel *C*). Each divalent cation was present at 20 mM. Each point is the mean of 4 experiments. The dashed line represents values predicted for K_B using the values in Table 2 and assuming that the apparent affinity of potassium for its cation site was not altered by the presence of the divalent cation

of potassium for its site was not altered by calcium (Fig. 6). The values in the presence of barium were not significantly different from those expected if the affinity of potassium for its site was not altered by the divalent cation (Fig. 6). With magnesium, calcium or barium the apparent dissociation constants describing the interaction between potassium and its site were (mM) 0.11 ± 0.04, 3.1 ± 0.4 and $0.27 + 0.09$, respectively. A similar effect of these divalent cations was observed using sodium, a monovalent cation which decreases the K_B for ouabain binding (Fig. 1). In the presence of magnesium (20 mM) the value for the dissociation constant describing the interaction between sodium and its site was significantly decreased (0.705 ± 0.078) and in the presence of calcium (20 mM) the value was significantly increased ($1.357 + 0.188$). Barium (20 mM) did not significantly alter the dissociation constant for sodium (1.020 ± 0.172).

Fig. 7 illustrates values of K_B at different lithium concentrations in the presence and absence of divalent cations. In the presence of magnesium as the lithium concentration increased, K_B increased, reached a maximum and then steadily decreased toward the same minimum value that was obtained without magnesium. A similar phenomenon was observed with 20 mM barium. In contrast to these effects, with 20 mM calcium, values for K_B at different lithium concentrations were significantly higher than those obtained in the absence of calcium. In addition, in the presence of calcium relatively low concentrations of lithium did not produce the initial increase in K_B which was observed with magnesium or barium.

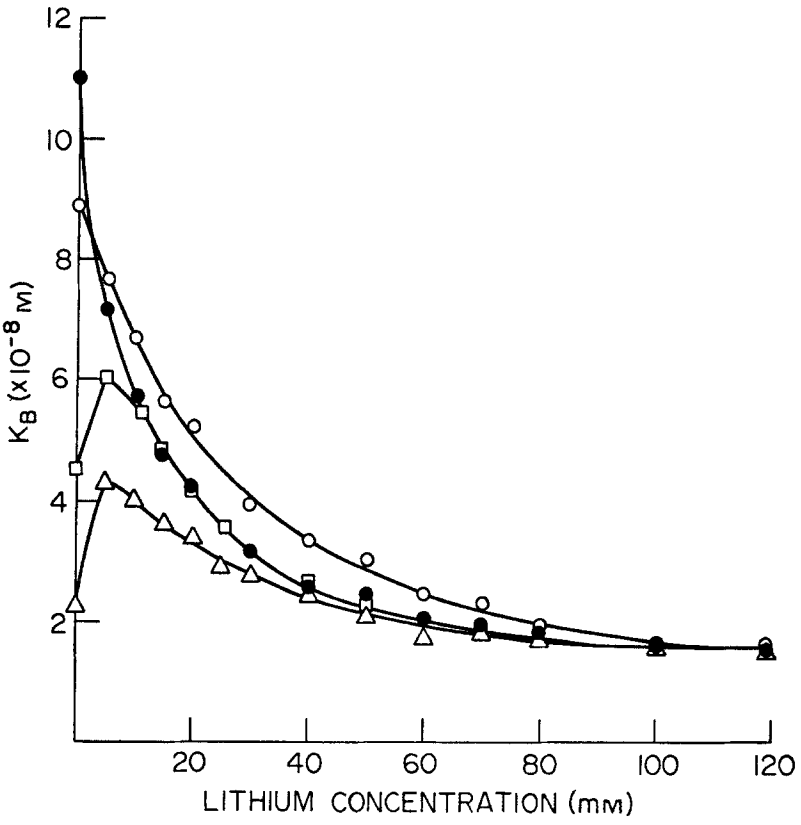

Fig. 7. Effect of lithium on the value of K_B for ouabain binding in the presence and absence (closed circles) of divalent cations. Calcium (open circles), barium (open boxes) or magnesium (open triangles) was present at 20 mM. Each point represents the mean of at least 3 experiments

Discussion

The present results indicate that monovalent and divalent cations act at sites which are functionally distinct from those to which ouabain binds and that these cations alter the apparent affinity but not the capacity of the erythrocyte membrane for glycoside molecules. Our previous finding of a direct correlation between ouabain binding to human erythrocytes and the reduction of potassium influx [10] plus the present results (Fig. 1 and Table 3) clarify and support the observation by Beaugé and Adragna [5] that ouabain inhibition of rubidium influx in human erythrocytes is dependent on the external concentrations of rubidium, sodium and ouabain. That is, rubidium reduces and sodium potentiates the effect of ouabain as a consequence of their altering the apparent affinity of the glycoside-binding site for ouabain.

The effects of extracellular cations on ouabain binding differ in several respects from effects of cations on Na,K-dependent ATPase and on the sodium-potassium pump. Our finding a single class of sites at which monovalent cations act to alter ouabain binding contrasts with the observations that there are two functionally distinct sites at which monovalent cations activate Na,K-dependent ATPase [33–35] and that at least two cations are thought to interact with the sodium-potassium pump during the transport process [13, 30, 31]. We observed mutual competitive interactions among the various monovalent cations in their ability to alter ouabain binding. Similar competitive behavior among different monovalent cations has been observed for activation of Na,K-dependent ATPase [35] and for the sodium-potassium pump [31]. Our results indicate that rubidium and potassium inhibit ouabain binding while cesium and lithium are stimulatory. In contrast, rubidium, cesium and lithium can each substitute for potassium in activating Na,K-dependent ATPase [31] and in stimulating the sodium-potassium pump [13, 30, 31]. None of the divalent cations studied appear to interact directly with the site at which monovalent cations act to influence ouabain binding; however, both magnesium and calcium (but not barium) can modify the effects of monovalent cations on ouabain binding. Neither extracellular calcium nor magnesium alter potassium influx in metabolically intact cells [11, 12, 24, 32]; however, both calcium and magnesium compete with sodium at the sodium activation site of Na,K-dependent ATPase [19–22, 32] and calcium can potentiate the effect of potassium at the potassium activation site [32].

Others, using erythrocytes [8, 9, 17, 18] as well as other cell types [3], have found that ouabain binding is reduced when a portion of the external sodium is replaced by cesium. Our results are consistent with these previous findings but indicate that the effect of cesium alone is to increase ouabain binding (Fig. 1, Table 3). These results are attributable to the cation site having a greater affinity for cesium than for sodium and to the glycoside-binding site having a lower affinity for ouabain in the presence of cesium than in the presence of sodium (Table 2).

Cesium has been interpreted to reduce "nonspecific" glycoside binding to human and sheep erythrocytes because cells incubated with cesium and ouabain bound fewer ouabain molecules but showed inhibition of cation transport comparable to that observed in a cesium-free, sodium-containing solution [8, 9, 17, 18]. In contrast, Baker and Willis [3], studying ouabain binding to HeLa cells, concluded that cesium inhibited specific ouabain binding; however, they made no direct comparison of cesium's effect on ouabain binding with its effect on potassium influx. The present results

indicate that cesium affects ouabain binding by acting at the same site and through the same mechanism as the other monovalent cations studied and that its effect differs from those of the other monovalent cations only in a quantitative manner. Our results also raised the possibility that the previously observed alteration in the relation between ouabain binding and cation transport when sodium was replaced by cesium [8, 9, 17, 18] might have reflected a primary effect of cesium on cation transport rather than on ouabain binding. To explore this possibility, we compared the effects of cesium on ouabain binding and on potassium influx with those of sodium (Fig. 8). With either cation, as ouabain binding increased potassium influx decreased. In erythrocytes preincubated without ouabain, replacing sodium by cesium reduced potassium influx by 50% and as ouabain binding increased, the magnitude of this difference diminished steadily. These results indicate that the change in the relation between ouabain binding and potassium influx observed in erythrocytes preincubated with cesium is attributable to a primary effect of cesium on the potassium transport mechanism instead of to a reduction in "nonspecific" ouabain binding.

Values for ouabain binding observed in the presence of both a monovalent cation and a divalent cation did not agree with values predicted by assuming that these two cations act at the same site (Table 3). This discrepancy suggests that the site for monovalent cations is functionally distinct from the site for divalent cations. Furthermore, in the presence of relatively high concentrations of monovalent cations, magnesium produced no significant change in ouabain binding (Fig. 4) and in the presence of relatively high concentrations of divalent cations, as the concentration of a particular monovalent cation was increased K_B approached the same final value as it did in the absence of divalent cations (Figs. 5 and 7). These observations indicate that when both the monovalent cation site and the divalent cation site are occupied, the affinity of the glycoside site for ouabain is the same as it is when only the monovalent cation site is occupied (*see* Fig. 4).

In addition to altering the affinity of the glycoside-binding site for ouabain, magnesium and calcium but not barium also altered the apparent affinity of the monovalent cation site for monovalent cations (Fig. 6).

Fig. 9 illustrates a hypothetical model which can explain the results which we have discussed thus far. This hypothesis assumes that the erythrocyte membrane contains a finite number of "receptor complexes" and that each receptor complex is composed of three functionally distinct sites: a monovalent cation site, a divalent cation site and a glycoside-binding site. The complex can exist in four functional configurations depending on whether

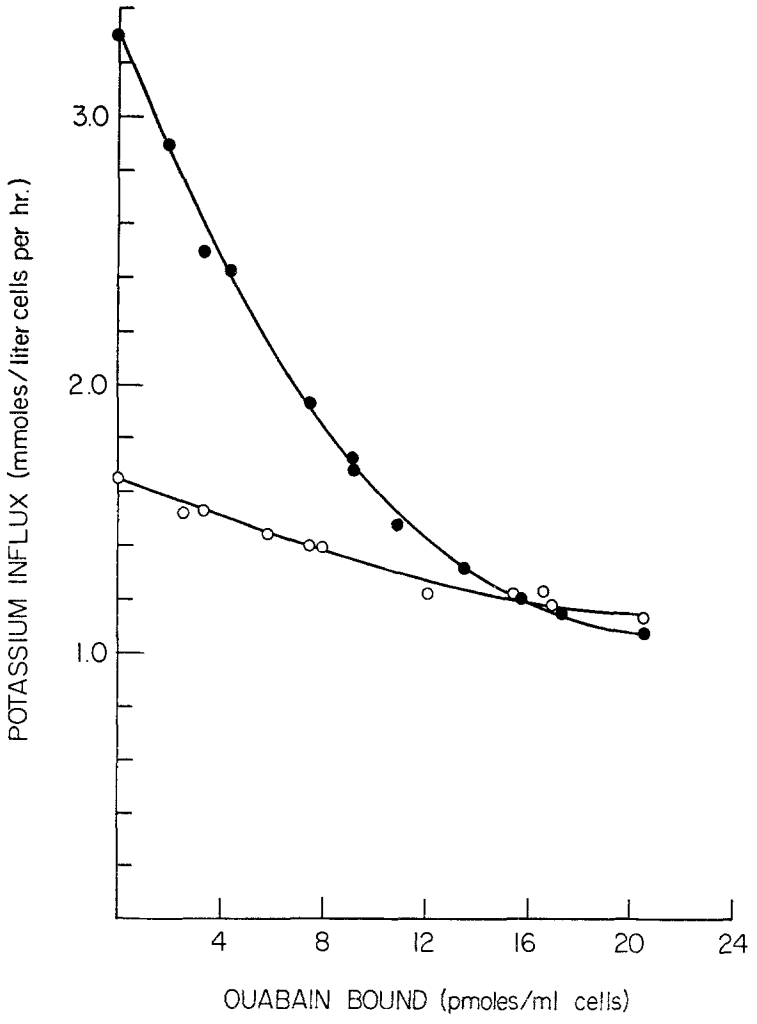

Fig. 8. Potassium influx as a function of ouabain bound for human erythrocytes. The cells were preincubated for 3 hr with different concentrations of ^{3}H-ouabain in solutions containing either 150 mM sodium (closed circles) or 150 mM cesium (open circles). At the end of the preincubation period samples were taken to determine ouabain binding. The remaining cells were washed 4 times with iced, isosmotic choline chloride and added to fresh incubation solution for the determination of potassium influx. The composition of the solution used to measure potassium influx was (mM): NaCl, 150; KCl, 15.9; Tris-HCl (pH 7.4), 10; glucose, 11.1. This experiment is representative of 3 others

none, one or both of the cation sites are occupied. The configuration of the complex as well as the particular cations which occupy the cation sites determine the affinity of the glycoside-binding site for ouabain. The values of $K_1 - K_3$, K_5 and K_8 for the functional interactions depicted in Fig. 9 are

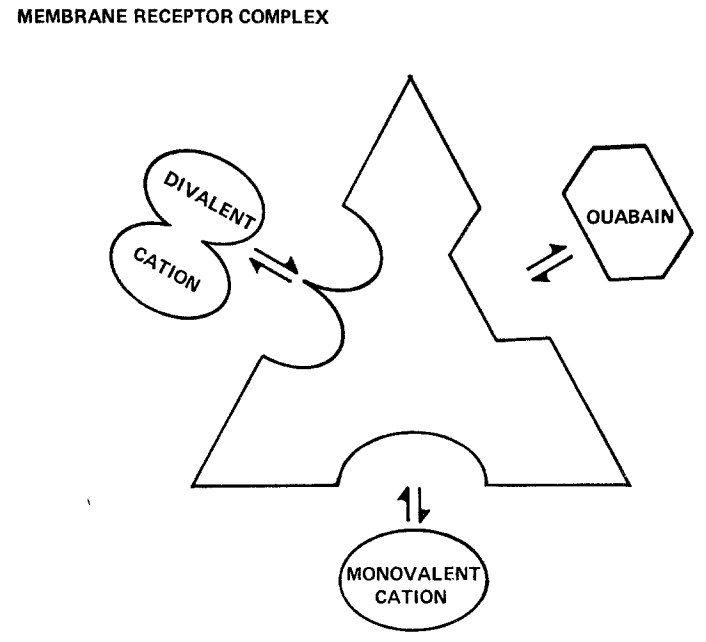

Fig. 9. *Upper portion* illustrates a hypothetical model to account for the effects of monovalent and divalent cations on ouabain binding to the human erythrocyte membrane. Ouabain binding is conceptualized as involving a membrane receptor complex having three functionally distinct sites (monovalent cation site, divalent cation site and glycoside-binding site) each of which can associate reversibly with a monovalent cation, a divalent cation and a ouabain molecule, respectively. The functional interactions of this complex are depicted in the *lower portion* where X, A, B and C represent receptor complex, ouabain, monovalent cation and divalent cation, respectively. K_i are the dissociation constants for the various substrate-complex interactions. The complex can exist in four distinct configurations (X, BX, XC and BXC) depending on whether none, one or both of the cation sites are occupied. The configuration of the complex as well as the particular cations which occupy the cation sites determine, in turn, the affinity of the glycoside site for ouabain. As is discussed in the text $K_4 = K_2$ for the various cations examined in the present study

given in Table 2. The values of K_7 for sodium and potassium have been given in the Results section of this paper and K_6 can be calculated from $(K_7 \cdot K_8/K_5)$.

The biphasic change in K_B produced by lithium in the presence of magnesium or barium (Fig. 7) suggests that lithium, unlike the other monovalent cations, can also interact with the divalent cation site. Since K_B increased with the addition of lithium, the affinity of the glycoside site for ouabain when lithium occupies the divalent cation site must be less than the affinity of the glycoside site when magnesium or barium occupy the divalent cation site. If this were not the case, replacement of magnesium or barium by lithium at the divalent site would produce either no change or a decrease in K_B. In the absence of divalent cations, the relation between K_B and the external lithium concentration (Figs. 1 and 7) can be explained satisfactorily in terms of lithium acting at a single site (the monovalent cation site) to alter the affinity of the glycoside-binding site for ouabain. That is, if lithium is also acting at a site other than the monovalent cation site, then the affinity of the glycoside site produced by lithium occupying this "other" site must not be significantly different from the affinity of the glycoside site observed in a solution containing 150 mM choline. Thus, in terms of its effect at the divalent cation site, adding lithium to a solution containing magnesium or barium is equivalent to removing divalent cations from the solution. Since this lithium-induced decrease in ouabain binding occurred at relatively low lithium concentrations, the affinity of lithium for the divalent cation site must be greater than its affinity for the monovalent cation site. From the data in Fig. 7, the value of the dissociation constant describing the interaction between lithium and the divalent cation site was 0.36 ± 0.09 mM (mean $\pm$ SD) and this value is significantly lower than the value for the interaction between lithium and the monovalent cation site (Table 2).

In the presence of calcium, addition of lithium did not produce the initial increase in K_B which was observed in the presence of magnesium or barium, and the values of K_B in the presence of calcium were higher than those in the calcium-free solutions. These results can be explained by postulating that calcium decreases the affinity of the monovalent cation site for lithium just as it decreases the affinity of the monovalent cation site for other monovalent cations (*see* Fig. 6). From the data in Fig. 7 the value of the dissociation constant describing the interaction between lithium and the monovalent cation site in a calcium-containing solution was 472 ± 138 mM and this value is significantly greater than the value of the dissociation constant in a solution free of divalent cations (Table 2).

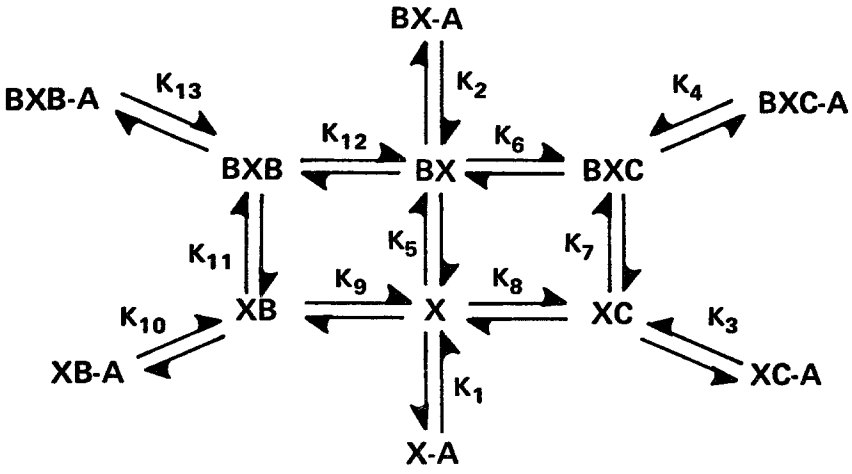

Fig. 10. Functional interactions of the membrane receptor complex illustrated in Fig. 8 in the presence of lithium. *X*, *A*, *B* and *C* represent receptor complex, ouabain, lithium and divalent cation, respectively. K_i are the dissociation constants for the various substrate-complex interactions. Since lithium can interact with the divalent cation site, the complex can exist in five distinct functional configurations (*X*, *XB*, *XC*, *BXB* and *BXC*). Since the affinity of lithium for the divalent site is greater than its affinity for the monovalent site, *BX* exists only as a hypothetical intermediate. The configuration of the complex as well as the particular divalent cation which occupies the divalent cation site determine the affinity of the glycoside site for ouabain. As discussed in the text, $K_{13} = K_4 = K_2$ and $K_{10} = K_1$. Furthermore, it has been assumed that association of lithium with the divalent site does not alter the affinity of the monovalent site for lithium; therefore, $K_{11} = K_5$ and $K_{12} = K_9$

Fig. 10 illustrates the functional interactions between lithium and divalent cations for the hypothetical model depicted in Fig. 9. Notice that because lithium can interact with the divalent cation site, the membrane receptor complex can exist in five different functional configurations. Since the affinity of lithium for the divalent site is greater than its affinity for the monovalent site, the receptor complex with only the monovalent site occupied by lithium (i.e., *BX* in Fig. 10) exists only as a hypothetical intermediate. The configuration of the complex as well as the particular divalent cation which occupies the divalent cation site determine the affinity of the glycoside site for ouabain.

The hypotheses depicted in Figs. 9 and 10 illustrate that although each cation studied altered the affinity but not the capacity of the erythrocyte membrane for glycoside molecules, the magnitude and relative direction of this change were the result of a complicated set of interactions involving the various cations and the erythrocyte membrane. There are certainly alternative proposals which could account for our findings; however, the

mechanism which we have proposed appears to be the simplest explanation which will account for our observations. This hypothesis should prove useful in studying the effects of cations on glycoside binding in other tissues and also facilitate the distinction between effects of cations on glycoside binding and their effects on glycoside-sensitive cation transport.

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