Invited Discussion

The Interaction between Tritiated Ouabain and the Na-K Pump in Red Blood Cells

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These comments are directed toward the nature of the interaction between cardiotonic steroids, such as ouabain, and the red blood cell plasma membrane. It has been known since 1953 (1) that one of the primary reactions associated with this class of steroids is their ability to inhibit the active transport of both Na and K in red cells as well as in a variety of other types of cells and tissues (2). These steroids display a very high degree of chemical specificity and act in rather low concentrations directly on the membrane. Their action on the red cell membrane is known to be asymmetric, acting only outside of the membrane (3), the side from which K is actively transported.

The membrane sites to which these steroids are bound are thought to be allosteric to the transport pathway, that is, that the cardiotonic steroids act as noncompetitive inhibitors of active transport (3). K, in addition to being actively transported, can antagonize directly the binding of these steroids to the membrane. Thus, when the concentration of inhibitor is low, K can control the rate or prevent the binding of glycosides by the membrane. It is of interest to mention the effect of Cs. While it is known that Cs can completely replace K in activating the Na-K pump, Cs unlike K, does not appear to alter or prevent the binding of cardiac glycosides to the membrane at pump-associated sites (3). We will now consider studies concerned with direct measurements of the binding of tritiated ouabain to intact red cells and their ghosts, carried out in collaboration with Christine J. Ingram (4) and Philip B. Dunham. Our interest in these studies is to determine not only the number of

glycoside-binding sites of the membrane but also to access the various factors which control the binding of glycosides to the membrane.

Our general experimental procedure is to incubate washed red cells (in this case human) at 37°C in an isotonic tris-buffered saline medium containing tritiated ouabain of high specific activity (approximately 515 mc/mM). In the experiments shown in Table I, portions of cells were removed at the indicated times and washed in the cold with ouabain-free medium. At each time period one portion of washed cells was reincubated at 37°C and K influx esti-

TABLE I

THE RELATION BETWEEN THE PER CENT INHIBITION OF PUMP FLUX OF K AND THE NUMBER OF MOLECULES OF OUABAIN BOUND PER HUMAN RED CELL

Washed red cells were incubated in a medium containing 153 mM NaCl and 17 mM tris buffer (pH 7.4) in the presence and absence of 6×10^{-9} M tritiated ouabain. Aliquots of the suspension mixture were removed at the indicated times, centrifuged, and washed. A portion of the cells was counted for tritiated ouabain content and the number of molecules bound per cell determined. The influx of K was measured on another portion of the washed cells which were reincubated in a medium containing 148 mM NaCl, 5 mM KCl, and 17 mM tris buffer in the presence and absence of added 1×10^{-4} M unlabeled ouabain. The ouabain-sensitive portion of K influx was taken as a measure of the pump flux of K. Per cent inhibition was determined from the difference between the pump flux of tritiated ouabain exposed cells to that of control cells treated in the same fashion. The results of two experiments, A and B, are presented.

	Inhibition		Molecules per cell	
Exposure	A	B	A	B
min	%	%		
30	35	40	70	79
120	75	85	165	152
240	82	89	173	165

mated using ^{42}K. We had previously shown in various types of control experiments, that essentially no adsorbed tritiated ouabain was lost as a result of the washing procedure. Another portion of the washed cells was processed immediately for counting tritium. Thus, for each time period we have an estimate of the per cent inhibition of the pump flux and the number of ouabain molecules associated with each cell under the same circumstance. The number of molecules of ouabain is calculated from the measured tritium content, the number of cells and the specific activity of the tritiated ouabain. The results of two different experiments are shown. It is apparent that uptake of ouabain increases with time and that this increase is associated with parallel increases in the inhibition of the pump. The purity of the tritiated ouabain was established by alteration of the specific activity (by the addition of unlabeled

ouabain) and by the action of K in preventing altogether the binding of tritiated ouabain.

If a higher concentration of ouabain is used than is indicated in Table I, or if the time of incubation is extended, the level of inhibition of the pump reaches, of course, 100 % but the uptake of ouabain continues far beyond the values shown here. This would indicate that there is a nonspecific uptake of tritiated ouabain accompanying the specific uptake of ouabain by pump-inhibited sites. The question is what is the extent of this nonspecific binding?

The total number of ouabain molecules that are bound to a single cell when the pump is 100 % inhibited averages about 200 molecules per cell. We have recently found that Cs appears to reduce or prevent the nonspecific binding of ouabain by the cells. Thus, cells incubated in a fashion similar to the ones shown in Table I, but in the presence of Cs, bind ouabain at about the same rate as seen here with comparable degrees of inhibition of the pump. However, even though the pump is inhibited to the same degree in the presence as in the absence of Cs, the amount of ouabain bound per cell is reduced to about one-half. This means that the previous number of 200 falls to about 100 ouabain-binding sites per cell and this number presumably still represents an upper limit. In terms of surface area, this would mean that there is on the average a little less than one ouabain-binding site per square micron of surface. It is not known how many pumps are associated with each glycoside-binding site but if there is a one-to-one correspondence, that is, one site per pump, then the turnover rate would be approximately 150 ions pumped per site per second.

While the previous results refer to human red cells, Dr. Dunham has recently carried out comparable experiments using two types of sheep red cells. These sheep cells are of special interest because, under the apparent control of a single gene (5), one type of sheep has red cells with a high K content while the other type has red cells with a low K content (see Table II). As previously reported (6), the magnitude of the pump flux in high K cells is several times larger than the pump flux in low K cells. This difference in pump rates is also paralleled by measurements of Na, K-ATPase activity (7). Curiously enough, as shown in Table II, this difference between the two types of red cells is also characteristic for the measured number of ouabain-binding sites. Thus, when the pumps are completely inhibited each high K type red cell binds, in the presence of Cs, about 40–50 ouabain molecules whereas the low K type red cell binds only 5–10. (The presence of Cs reduces the number of glycoside-binding sites in HK and LK type cells to the same extent; i.e., approximately seven sites per cell in both instances.) Thus, from the values presented in Table II, the ratio of pump rates to binding sites in HK compared with LK is 7.7 and 7.0, respectively. In terms of surface area, since the sheep cell is smaller than the human cell, the number of glycoside-binding sites for

the high K type is very similar to the value given before for human. Obviously, the low K cell compared with the high K type has about 1/7th the number of glycoside-binding sites per square micron of surface and based on a correlation of one site per pump, these data indicate that while the two cell types have quantitatively a different number of pumps they have completely comparable turnover rates.

Finally, I would like to mention some other types of results we have obtained concerning various factors which control the binding of glycosides to the membrane. For this work we have used hemoglobin-free ghost membranes

TABLE II

THE RELATION BETWEEN THE PUMP FLUX OF K AND THE NUMBER OF OUABAIN-BINDING SITES IN HK AND LK RED CELLS FROM DORSET SHEEP

$(K)_i$ and $(Na)_i$ represent the cellular concentrations of K and Na, respectively in mM/liter cells. The values in parentheses give the number of different sheep studied. The symbol, $^iM_K^P$, represents the pump influx of K in mM/liter cells × hour. Variance is expressed as standard deviation. The cells were incubated in a medium containing 128 mM NaCl, 25 mM CsCl, and 17 mM tris buffer in the presence and absence of various concentrations of tritiated ouabain. The uptake of tritiated ouabain was measured at various intervals during incubation on washed cells. Influx of K was also measured on the washed cells, removed at various intervals, by reincubation in 143 mM NaCl, 10 mM KCl, and 17 mM tris buffer in the presence and absence of added 1×10^{-4} M unlabeled ouabain. The ouabain-sensitive portion of K influx represents $^iM_K^P$. The number of sites per cell represents the number of molecules of ouabain associated with each cell when the pump flux of K is inhibited 100%.

Type	$(K)_i$	$(Na)_i$	$^iM_K^P$	Sites per cell
HK (5)	76.9	27.2	0.89 ± 0.11	43.7 ± 12.0
LK (6)	14.2	95.4	0.115 ± 0.022	6.2 ± 2.6

prepared from human red cells by successive osmotic hemolysis which were subsequently frozen and thawed to remove any permeability barrier to added ions or substrates. The ghosts are treated in much the same fashion as described for intact cells, the ghosts being exposed to tritiated ouabain under different conditions prior to washing in the cold and counting. When appropriate, Na, K-ATPase activity was measured in place of determinations of unidirectional flux.

Many of the results that are reported below are not very different in kind from those observed by others on microsomal preparations isolated from calf heart (8, 9), brain (10), and the electric organ of the electric eel (11). But it should be kept in mind that it is only in the red cell that the direction of active transport and membrane orientation are known.

Similar to the results of others we have found, as shown in Table III, that the presence of ATP promotes the binding of ouabain to the ghost membrane. But more important at the moment, this table also shows the procedure used to estimate the extent of binding of ouabain by ghosts under various conditions. The method is based on the difference in the amount of binding observed between exposure in paired flasks to high and low specific activity tritiated ouabain. The baseline amount of tritiated ouabain associated with

TABLE III

THE EFFECT OF ATP ON THE UPTAKE OF TRITIATED OUABAIN (T-OUABAIN) BY HUMAN RED CELL GHOSTS

Ghosts are incubated for 30 min at 37 °C in a medium containing 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM tris buffer, and tritiated ouabain. Ghosts are added to paired flasks in the presence and absence of added unlabeled ouabain. The number of molecules of ouabain bound per ghost is calculated from the difference between the dpm obtained from each set of paired flasks. While the number of molecules of ouabain bound per ghost under optimal conditions compares favorably with the results obtained for intact cells, the values so determined in ghost are not as reliable because of the inaccuracy in the estimate of the number of ghosts.

Incubation medium	dpm/0.1 ml ghosts	Molecules per ghost
T-ouabain (1.3×10^{-7} M)	137	4
T-ouabain (1.3×10^{-7} M) + ouabain (10^{-4} M)	103	
T-ouabain (1.3×10^{-7} M) + ATP (0.002 M)	2006	156
T-ouabain (1.3×10^{-7} M) + ATP (0.002 M) + ouabain (10^{-4} M)	157	

the ghosts at low specific activity (i.e. tritiated ouabain and unlabeled ouabain) is always low and presumably represents trapped ouabain. The results presented in Tables IV and V are expressed in terms of the calculated molecules bound per ghost utilizing this difference procedure. It should be stated that the characteristics already described for ouabain binding to intact cells are paralleled in ghosts since the ATP-promoted binding of ouabain can be completely prevented by K and that the Na, K-ATPase activity, assayed after exposure to tritiated ouabain and after washing with ouabain-free solutions, was inhibited by binding promoted by ATP in contrast to its control (no ATP).

Table IV surveys the medium conditions necessary for ouabain to be bound by the membrane. There are more interesting differences when the requirements for glycoside binding are compared with the known properties of the Na, K-ATPase. This is so particularly with the role of added Mg and Ca and, as we will see below, with the specificity of the nucleotide. It is apparent that a smaller but significant amount of binding occurs without added Mg even in the presence of additional EDTA. The extent of this binding in the presence or absence of added Mg is unaffected by the addition of 5 mM Na F. Ca has no effect on the binding of glycoside to the membrane when added alone or in the presence of Mg.

TABLE IV

THE EFFECT OF MEDIUM COMPOSITION ON THE BINDING OF OUABAIN BY GHOSTS

Ghosts were incubated for 30 min at 37°C in the complete medium and in media in which the indicated deletions were made prior to washing and counting. The complete medium contained 40 mM NaCl, 2 mM Tris ATP, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM tris buffer, and 2.4×10^{-7} tritiated ouabain. The concentration of the various additions to the medium was 2.25 mM EDTA, 40 mM choline chloride, or 1.25 mM $CaCl_2$.

Medium conditions	Molecules per ghost
Complete	139
Minus ATP	6
Minus Mg	38
Minus Mg + EDTA	30
Minus Na	105
Minus Na + Choline	110
Minus Mg + Ca	45
Plus Ca	128

In contrast to these results, added Mg is an obligatory requirement for Na, K-ATPase activity. Ca cannot replace Mg and acts to inhibit the Na, K-ATPase activity in the presence of Mg (12). While Na is neither required nor influences the binding of glycoside to the membrane, Na, K-ATPase activity obviously requires the combined presence of Na and K. It is possible to get completely comparable data on glycoside binding in the presence of Na and K at higher concentrations of tritiated ouabain but the relative effects of Mg and Ca as shown here remain the same.

Table V shows the relative effects of different nucleotides in their ability to promote the binding of glycoside to the membrane. It is clear that ITP, CTP, and ADP are just as effective as ATP in promoting the binding of ouabain to the membrane. This is so even in concentrations as low as 1 μM. Completely comparable results have also been obtained with UTP, GTP, dATP, and dADP in that all promote glycoside binding essentially to the same extent.

Removal of Na does not alter either the nucleotide effects or their concentration dependence in promoting the binding of ouabain. Thus, for glycoside to be bound it appears that essentially any nucleotide-triphosphate and most nucleotide-diphosphates will do the job. This is very odd since the pump as well as the Na, K-ATPase has an exclusive dependence on purine nucleotides greatly preferring ATP over ITP. Pyrimadine nucleotides are essentially inactive, not being broken down at all (12). There are a number of other conditions, such as inorganic phosphate (in the absence of Na), which will promote the binding of glycoside to the membrane but limitations of time prevent their discussion.

TABLE V

THE EFFECT OF VARIOUS NUCLEOTIDES ON THE BINDING OF OUABAIN BY GHOSTS

Ghosts were incubated for 30 min at 37°C prior to washing and counting in a medium which contained 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 10 mM tris buffer, 2.4×10^{-7} M tritiated ouabain, and the indicated concentrations of the different nucleotides.

Nucleotide added	mM/liter	Molecules per ghost
ATP	2.0	174
	0.01	152
	0.001	75
ITP	2.0	189
	0.01	160
	0.001	60
	0.0001	15
CTP	2.0	168
	0.01	161
	0.001	90
ADP	2.0	184
	0.01	144
IDP	0.01	13
AMP	2.0	7

How can these various results be interpreted? It seems to me that what is basically involved is a change in membrane conformation. Given the membrane orientation and the boundary conditions that are known to exist in the intact cell, the emerging picture is the following: Nucleotide-triphosphate, present on the inside of the membrane is bound at the specific locus of the Na, K-ATPase. As this nucleotide is bound, the membrane in this region alters its conformation such that the outside surface is now susceptible for attack by glycoside. If glycoside is bound, the membrane is held in this conformation and the pump is inhibited. In the absence of glycoside the conformation cycle is completed by breakdown of its bound substrate with the concomitant exchange of external K for internal Na. The conformation change itself that

leads to glycoside binding can be antagonized by external K and does not appear to require the formation of any phosphorylated intermediate since the conformation can be induced in the absence of Na and Mg and by a variety of nucleotides in very low concentration.

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Discussion from the Floor

Dr. George Siegel (Mt. Sinai School of Medicine): I should like first to confirm some of Dr. Post's findings. Dr. Albers and I have made microsome preparations from electroplax and cat brain which when treated with ouabain do incorporate inorganic phosphate. And upon digestion of the acid-stable phosphoryl protein, the electrophoretic mobilities of the phosphopeptides are very similar to those obtained from $AT^{32}P$ labeling. And in fact the patterns are quite similar to Dr. Post's.

In view of this, I'd like to ask of Dr. Glynn and Dr. Post, which of the inorganic-phosphate incorporations may be specific for $(Na^+ + K^+)$-ATPase–that which is obtained only in the absence of ouabain in the red cells which appears in ATP, or that which is obtained only in the presence of ouabain in these microsomal preparations? How would you reconcile these two phenomena as both being specific for the pump ATPase?

Dr. Post: The conditions are quite different. In the intact red cell there are large cation gradients against the direction of the pump. In principle these can supply energy for reversal of the pump and synthesis of ATP from ADP and inorganic phosphate.

In the broken membrane there is no source of energy from gradients. The phosphorylated ouabain-enzyme complex has not reacted with ADP in our experiments, so that ATP synthesis from this inhibited form does not appear to be possible. I'm inclined to interpret the phosphorylation of the ouabain-enzyme complex as analogous to a reversal of the dephosphorylation step in the normal reaction sequence. It think it is up to the membrane ATPase people to try to work out a sequence of conditions by which they, too, might get reversal all the way back to ATP if they wish to correlate their experiments with those in the more physiologic system. I believe that both phosphorylations from inorganic phosphate are aspects of the same transport system under different circumstances.

Dr. Glynn: I don't really have an answer for this question. It is surprising that you get incorporation of inorganic phosphate when ouabain is present, but it is perhaps less surprising if you accept that it is wrong to think of the transport ATPase as completely inactive in the presence of ouabain. We know that potassium influx still shows a small saturatable component in the presence of sufficient ouabain to give maximal inhibition. Under similar conditions potassium efflux, too, is still affected by the concentration of external potassium. So it looks as though ouabain does not completely stop everything but allows some activity of a kind that we do not understand. This does not explain the phosphate incorporation, but the existence of another fact of the same kind makes the two together less surprising, in a way, than either alone.

Dr. Arthur Kahlenberg (Lady Davis Institute for Medical Research, Montreal): First, in reference to Dr. Glynn's remarks that, to accept Robinson's interpretation of his results in terms of conformational effects (*Biochemistry.* 1967. **6**:3250), one would have to demonstrate an inhibition of the Transport ATPase by a high or low ATP concentration, I would like to call his attention to a recent publication by Baskin and Leslie (*Biochim. Biophys. Acta.* 1968. **159**:509). These authors reported that concentrations of ATP above 1.5 mm do indeed inhibit the transport ATPase.

Secondly, I would like to ask Dr. Post a question: to support your proposal that there are two forms of a phosphorylated intermediate, as demonstrated by their different sensitivity to ouabain, would you not be required to demonstrate this difference in the presence of the same test materials? It seems to me that one way of achieving this would be to test the ADP inhibition of $E_1 \sim P$ and $E_2 \sim P$ since your results indicate that $E_2 \sim P$ may be rendered sensitive to ADP at low magnesium concentrations.

Dr. Glynn: I said, I think, that the point needed further investigation and I am delighted that it has already been investigated. I am not sure that inhibition by excess ATP convinces me that we are necessarily dealing with an allosteric reaction but it does dispose of my second argument against allostery.

Dr. Post: The experiment at low magnesium concentration was not interpreted as showing that E_2-P became sensitive to ADP. It was merely taken as evidence that some portion of whatsoever phosphorylated forms there were had an E_1-P sort of reactivity. It was not considered that any E_2-P in that mixture would run backward to E_1-P at a low magnesium ion concentration. E_2-P has always been insensitive to ADP regardless of the addition of Mg^{++} or of EDTA with the ADP.

Dr. I. Herbert Scheinberg (Albert Einstein College of Medicine, New York): I'd like to ask Dr. Post whether or not the difference between the three sodiums that go out and the two potassiums that go in is measured directly, or whether it is an osmotic consequence of the freed phosphate ion? And I wanted to ask Dr. Hoffman whether the sheep cells which contain only 12 mM potassium have the difference made up by sodium or are hypo-osmolar compared with normal sheep cells.

Dr. Post: The stoichiometry of the pump has been directly measured in intact red cells and in ghosts. Dr. Glynn's and three other laboratories have confirmed it to one extent or another. You have to allow, plus or minus 20% on that 3 to 2, but it is directly measured.

I might throw in the comment that there is a lot of evidence for electrogenic pumps, so that the pump itself may really be electrogenic.

Dr. J. H. Copenhaver (Dartmouth College, New Hampshire): Although you talk about ouabain-inhibiting sites, there may be other types of sites on the enzyme specific for the glycosides. You must remember that very small levels of the glycosides will stimulate this ATPase. It may be that the stimulation effect of the glycosides on this enzyme is related to the cardiotonic effect of the drugs and that such effect is exerted at another site on the enzyme.

Dr. Hokin: I think some people would argue that the stimulation of the Na-KATPase by very low concentrations of cardiotonic steroids is responsible for the cardiotonic action of these drugs. However, I would agree that there may be more

than one site on the enzyme for cardiotonic steroids and that the stimulation of the Na-KATPase by cardiotonic steroids may be exerted at a different site from the one where the drugs inhibit the enzyme. With the present purity of the enzyme preparation there is clearly more labelling with the site-directed alkylating agents than can be accounted for by labeling at one site on the Na-KATPase. However, with nonalkylating steroids we label between one and two sites per mole of Na-KATPase (based on the number of L-glutamyl-γ-phosphate residues in the preparation), which may well turn out to be one site as purification proceeds.