PRELIMINARY NOTE

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On the number of sodium pumping sites in cell membranes

The fact that inhibition of the Na^+ pump by cardiac glycosides is highly specific and, in most tissues, is only very slowly reversed provides a relatively simple means of measuring the number of pumping sites per cell. Thus HOFFMAN AND INGRAM[1] and ELLORY AND KEYNES[2], both working with red cell ghosts, have used labelled glycosides to measure the number of binding sites per cell. Their results are striking because they find only about 200 binding sites per cell or about 1 pumping site per μ^2 of cell surface.

In view of the fact that the ouabain-sensitive fluxes of Na^+ and K^+ in red cells are appreciably lower than in most other tissues, it seemed of interest to repeat these experiments on more active cells. In order to do this it was necessary to find a relatively uniform preparation of known cell density which also had a high sensitivity to

TABLE I

BINDING OF [^{3}H]OUABAIN TO CELLS OF VARIOUS TYPES

Suspensions of cultured cells ($1 \cdot 10^6$ cells/ml) from Flow Laboratories, (Irvine, Scotland) were incubated with [^{3}H]ouabain (New England Nuclear) in the presence of 6 mM K^+, either in culture medium (Eagles) or in phosphate-buffered artificial saline. The labelled cells were washed 4 times in 10-ml aliquots of ice-cold saline containing 0.1 mM cold ouabain, dissolved in Nuclear Chicago Solvent and counted in a liquid scintillation counter using a toluene–2.5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl-2)benzene scintillation fluid. Both media gave similar results, and the values listed are the maxima obtained during a 2-h exposure to 0.1, 1.0 or 10 μM glycoside. Tissue slices and red cells were incubated in phosphate-buffered saline. Temp., 35°. Where possible results are expressed as mean ± S.E. of the mean with the number of experiments in parentheses. For comparison, the binding to human red cells is 100–200 molecules/cell[1,2]. The results with cultured cells have been converted to molecules/mg on the assumption that $1 \cdot 10^6$ cells have a wet wt. of 2 mg. These calculated figures are shown in italics. 1 mg of packed guinea-pig red cells contained $1.4 \cdot 10^7$ cells.

Species	*Cell type*	*Glycoside bound*					
		$1 \cdot 10^5$ molecules/cell			*$1 \cdot 10^{11}$ molecules/mg wet wt.*		
	Ouabain concn.:	*0.1 μM*	*1 μM*	*10 μM*	*0.1 μM*	*1 μM*	*10 μM*
Human	HeLa (S-3)	3.2 ± 0.3 (16)	7.3 ± 1.1 (6)	—	*1.6*	*3.7*	—
	Heart (Girardi)	2.6 (2)	5.0 (1)	—	*1.3*	*2.5*	—
	Sternal marrow (Detroit)	3.9 (2)	—	—	*2.0*	—	—
Rabbit	Kidney (RK-13)	1.8 (1)	4.3 (1)	4.6 (1)	*0.9*	*2.2*	*2.3*
Guinea pig	Kidney (primary culture)	1.2 (2)	5.1 (3)	4.9 (1)	*0.6*	*2.6*	*2.5*
	Kidney slices	—	—	—	2.3 (1)	10.3 (5)	
	Brain slices	—	—	—	—	14.0 (2)	—
	Liver slices	—	—	—	0.2 (2)	1.6 (3)	—
	Red blood cells	0.0007 (1)	0.0012 (2)	—	0.0098 (1)	0.0166 (2)	—

cardiac glycosides. Suspensions of various tissue culture cell lines were chosen as best satisfying these requirements.

Table I summarises results obtained with a variety of cultured cells. It will be seen that the binding to cultured cells is 3–4 orders of magnitude higher than that to fresh human and guinea-pig red blood cells. Although the actual numbers may be subject to some error, the difference is striking and poses the obvious question whether the binding of glycoside to tissue culture cells is really measuring pumping sites. We think it is for the following seven reasons.

(1) At low glycoside concentrations provided there is enough glycoside to inhibit the Na^+ pump, the final level of binding is largely independent of the concentration of glycoside used. The rate of binding is slower at low glycoside concentrations. In the absence of glycoside, binding can be reversed and the rate constants for binding and debinding are consistent with the measured inhibitor constant.

(2) The rate of binding is very temperature-dependent and in HeLa cells is increased 5-fold on raising the temperature from 25 to 35°.

(3) Binding is very sensitive to external K^+. In HeLa cells exposed to 0.2 μM ouabain, raising the K^+ concentration from 0 to 10 mM reduces the rate of glycoside binding to less than one tenth of its rate in the absence of K^+. The rate is even slower in the presence of 50 mM K^+ where after a 2-h exposure to 0.2 μM ouabain, binding is only about 10 % of that in the absence of K^+. These effects are due to the presence of K^+ and not to the concomitant reduction in external Na^+ because the rate and extent of binding is quite normal if the Na^+ is replaced by choline instead of K^+. It is well known that K^+ tends to counteract the inhibitory action of the cardiac glycosides on the Na^+ pump, and the striking effects of external K^+ on glycoside binding suggest that the bulk of the glycoside is binding to Na^+ pumping sites. The rate of glycoside debinding is largely independent of K^+ concentration.

(4) The rate and extent of binding is reduced in Na^+-free choline media, under anaerobic conditions and in the presence of CN^-. These observations suggest that at least part of the glycoside is binding to pumping sites because in intact squid axons BAKER AND MANIL[3] have shown that the rate of action of glycosides is slowed in CN^--poisoned cells; and on the isolated ($Na^+ + K^+$)-activated ATPase, SCHWARTZ *et al.*[4] and ALBERS *et al.*[5] have shown that glycoside binding is speeded up in the presence of Na^+ and ATP.

(5) The binding is associated with the cell surface and is not lost on lysis of the cells. The binding of ouabain to an isolated membrane preparation from HeLa cells is dependent on the presence of Na^+ and ATP, and the final level of binding is similar to that obtained with intact cells. The binding is reduced by K^+ and by iodoacetamide (5 mM).

(6) Once bound, the tracer can be released by warming to 70°, by treatment with 5 % (w/v) trichloroacetic acid, by exposure to 6 M urea or by extraction with ethanol. All these conditions are reported to displace ouabain from binding to Na^+ pump sites[2]. The ease of release argues against incorporation of the glycoside molecule into some macromolecular constituent of the cell.

(7) The uptake of tracer is reduced in the presence of 0.1 mM cold ouabain; but the total number of molecules bound per cell is increased, possibly due to non-specific uptake.

Taken together, the above results suggest that the bulk of the glycoside binding is associated with pumping sites, and estimates of the number of pumping sites per

cell seem unlikely to be in error by more than a factor of two. As this number is so high ($1 \cdot 10^5$–$5 \cdot 10^5$ sites/cell), it seemed of interest to make comparable measurements on uncultured tissues obtained direct from a living animal. Slices of guinea-pig kidney cortex were used, and the binding was compared on a wet weight basis to that found for cultured cells from the same organ. Both the absolute levels of binding and the properties of the binding mechanism were very similar. Thus the binding to guinea-pig kidney slices averaged $1 \cdot 10^{12}$ molecules/mg (Table I) and was markedly reduced by external K^+, in the presence of cold ouabain, in Na^+-free media, under anaerobic conditions and at 25°.

In order to discover whether the number of pumping sites per cell is particularly high in the kidney, we also made a few measurements on slices of guinea-pig liver and brain. Using 1 μM ouabain, the binding per mg wet wt. was $1.4 \cdot 10^{12}$ molecules for brain and $1.6 \cdot 10^{11}$ for liver. By comparison, red cells bind $1.7 \cdot 10^9$ molecules/mg wet wt. It thus seems that the red cell is the exception rather than the rule and that most cell membranes have a rather high density of Na^+ pumping sites. The recent experiments of O'DONNELL AND ELLORY[6] on mammalian sperm show that this tissue binds about $1 \cdot 10^4$ glycoside molecules/cell which is again two orders of magnitude higher than the red cell.

It is instructive to use the numbers in Table I to calculate the molecular turnover at Na^+ pumping sites in different tissues. When compared with the data of BONTING *et al.*[7] for the ouabain-sensitive ATPase in the same tissues, the turnover rates for red cells and slices of kidney, brain and liver all fall within the range 8000–13000 per min. A similar calculation can be made for HeLa cells using the initial rate of loss of K^+ following exposure to 10 μM ouabain. As the cell K^+ normally remains constant, provided that the glycoside simply stops the Na^+ pump, the initial rate of K^+ loss ($1 \cdot 10^{-2}$ pmoles/cell per min) should equal the steady state rate of K^+ uptake in the absence of glycoside. Assuming 2 K^+: $\sim$ P, and $5 \cdot 10^5$ pumps/cell, the turnover rate is 6000 min^{-1}. These values fall within the range 5000–15000 min^{-1} obtained by other workers[2] and add further support to the conclusion that the high binding of ouabain really reflects a large number of pumping sites per cell. It is difficult to assess the area of surface membrane per cell; but for HeLa cells, assuming a membrane area of 1000 μ^2, $5 \cdot 10^5$ pumping sites/cell and a cross sectional area per pump of 5500 A^2 (ref. 8) at least 3 % of the cell surface must be Na^+ pump sites. ALBERS *et al.*[5] estimate that Na^+ pump sites comprise 25 % of their isolated membrane, prepared from electric organ.

This high density of pumping sites raises two interesting possibilities: (1) changes in membrane conformation might be detectable during pumping in intact cells and (2) substances which interact with the membrane between the pump sites may alter the rate of pumping. In view of the wide variation in the number of pumping sites in different cells, it would be interesting to investigate what factors control both the absolute number of pumping sites in the cell membrane and their activity at any particular time.

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