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(Na, K)ATPase Activity in Membrane Preparations of Ouabain-Resistant HeLa Cells†

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ABSTRACT: Membrane preparations from two independent ouabain-resistant HeLa cell clones, HI-B1 and HI-C1, each appear to contain two species of (Na, K)ATPase. Two-thirds of the total (Na, K)ATPase in each mutant is indistinguishable from the enzyme in preparations of wild type cells with respect to ouabain binding, ouabain inhibition of (Na, K)ATPase activity, and dependence of ATP hydrolysis on Na, Mg, K, and ATP concentration. The remaining (Na, K)ATPase activity in the mutants is up to 1000 and 10 000 times, respectively, more resistant to ouabain than wild type enzyme. Resistance results from a lower affinity of the mutant enzymes for the inhibitor. The presence of Na, K, or Mg has little or no effect on the degree of resistance expressed by the mutant enzymes, although the resistance of the wild type enzyme varies 400-fold in the presence of different ligands. Incubation with 5×10^{-8} M ouabain abolishes the activity of the wild type enzyme without affecting the activity of the resistant enzymes. Using this procedure we compared the parameters of ATP hydrolysis via the resistant and wild type enzymes. Ouabain-resistant (Na, K)ATPase of HI-C1 has an apparent $K_{0.5}$ for potassium 3-4 times higher than that of either wild type enzyme or the resistant enzyme of HI-B1.

Mutants resistant to ouabain, a specific inhibitor of the plasma membrane (Na, K)ATPase, can be selected in a single step from HeLa cell lines (Baker, 1976). Dose-response curves describing ouabain inhibition of K influx suggest that mutant clones contain two types of K transport site which differ in their sensitivity to the inhibitor. The pattern of inhibition observed with wild type cells is consistent with the presence of a single class of transport site. The results of binding studies with the resistant HeLa cells also suggest that they contain two classes of ouabain binding sites, one similar to that in wild type cells and one with less affinity for the inhibitor. For the single-step mutants that have been examined, there appear to be two wild type sites for each ouabain-resistant one.

In this paper we describe the characteristics of the (Na, K)ATPase in membrane preparations of the wild type and two different ouabain-resistant clones of HeLa cells.

Experimental Procedure

Cell Lines, Media, and Growth Conditions. "Wild type HeLa" designates a clone, isolated in this laboratory, from HeLa cells (CCL 2) obtained from the American Type Culture

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Collection. The ouabain-resistant cells used in this study were obtained from wild type HeLa by the following procedures. HI-B1: an inoculum of 10^4 wild type HeLa cells was grown to approximately 2×10^6 cells and then treated, without mutagenesis, with 2×10^{-7} M ouabain. A single colony was obtained from this selection and this colony was recloned. HI-C1: an inoculum of 10^4 wild type HeLa cells was grown and mutagenized with ethylmethanesulfonate (100 μg/mL). Following a period for phenotypic expression, the culture was treated with 2×10^{-7} M ouabain. Four colonies were obtained from approximately 2×10^6 cells; these were grown and cloned. Cells were grown in MEM Alpha Modified (KC Biological Inc. LM-175) plus 5% fetal bovine serum (Flow Laboratories) in plastic roller bottles at 37 °C.

Chemicals. [γ-^{32}P]Adenosine 5′-triphosphate (2–10 Ci/mmol), [G-^{3}H]ouabain (12 Ci/mmol), and [G-^{3}H]deoxycholate (2 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Adenosine 5′-triphosphate, ouabain, and sodium deoxycholate were purchased from Sigma Chemical Co., St. Louis, Mo.

(Na, K)ATPase Preparation. Growth medium was decanted from the cells which were then washed once in phosphate-buffered saline and harvested by scraping. The cells were then washed twice with phosphate-buffered saline, once with 0.25 M sucrose, 1 mM Tris–EDTA, pH 7.4, resuspended in 1 mM Tris–EDTA, pH 7.4 (0.5 g wet weight cells/mL), and left on ice for 20 min. At this point freshly prepared sodium deoxycholate (5%) was added to a final concentration of 0.35% (w/v). The cells were kept on ice for 20 min with constant stirring of the suspension, which was then centrifuged at 10 000*g* for 10 min at 4 °C. The supernatant was removed and the pellet washed once with 1 mM Tris–EDTA, in volume equal to that employed above. The two supernatant fractions were pooled and centrifuged at 77 000*g* for 12–16 h at 4 °C. The supernatant fluid was removed and the pellet taken up and homogenized in 1 mM Tris–EDTA, pH 7.4. This material was stored on ice at approximately 5 mg of protein/mL. (Na, K)ATPase activity in this preparation is 0.125 enzyme unit/mg of protein (one enzyme unit effects the release of 1 μmol of phosphate per min at 37 °C); 18 enzyme units were obtained from approximately 5×10^9 cells. Dounce homogenization of the deoxycholate-treated preparation prior to the first centrifugation had no effect on the yield of (Na, K)ATPase; however, this additional step resulted in preparations of somewhat lower specific activity.

This unorthodox method of preparation was devised to circumvent the following problem: when HeLa fibroblasts were lysed by either homogenization or nitrogen cavitation, large membranous sheets were formed which sediment at 1000*g*. This material, containing most of the (Na, K)ATPase activity, is grossly contaminated with unbroken cells, nuclei, and large organelles. The enzymatic activity in this fraction was unstable to both storage and attempts at purification. (Na, K)ATPase activity in the deoxycholate extract described above is stable for more than 30 days when stored on ice.

Assay of (Na, K)ATPase Activity. The procedure used was adapted from that of Lane et al. (1973). In the standard assay, activity was determined at 37 °C in 1 mL of medium containing 30 mM histidine (adjusted to pH 7.1 with Tris), 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, and 1 mM [γ-^{32}P]-ATP (5×10^4 cpm/μmol). These concentrations of the respective ligands were found to be optimal for (Na, K)ATPase activity in preparations of HeLa cells in experiments in which the concentrations were varied as follows: Na, 5–200 mM; K, 0.5–20 mM; Mg, 0.1–15 mM; ATP, 0.005–5 mM. The deoxycholate preparation was added to a final concentration of 5×10^{-3} to 1.0×10^{-2} EU/mL and the assay period was 8 min. The assays were terminated by addition of 3 mL of ice-cold medium containing 100 mM HCl, 1 mM orthophosphate, 1 mM pyrophosphate, 0.6 mg of bovine serum albumin, and 120 mg of activated charcoal. After 30 min on ice the samples were filtered and the filters washed with 2×3 mL of 0.01 M HCl, 1 mM orthophosphate (Dunham and Hoffman, 1970). Under these conditions (Na, K)ATPase activity proceeds linearly with respect to both protein concentration and time. Cerenkov radiation of the samples was determined in a Beckman scintillation counter LS-230. All results are corrected by subtracting the ATPase activity measured in the absence of Na and K. This Mg-dependent ATPase activity varied with the age of the preparation, from 15% of the total ATPase activity in fresh preparations, to zero in preparations stored on ice for 3 weeks or longer. The results presented are the average of duplicate determinations which differed by <5%.

For those experiments in which the enzyme preparation was preincubated with ouabain and one or more of the ligands included in the standard assay mixture, the assay mixture was prepared in such a way that the final concentration of each ligand was identical with that described above. Measurements of the dependence of enzymatic activity on the concentration of a specified ligand were performed by varying the concentration of that ligand over the range of concentrations described in the text; the other ligands were present at the concentrations described above.

Assay of [^{3}H]Ouabain Binding. Activity was determined at 37 °C in 1 mL of buffer containing 30 mM histidine (adjusted to pH 7.1 with Tris) and 5 mM $MgCl_2$. The deoxycholate preparations were added to a final concentration of 5×10^{-3} EU (40 μg of protein) per mL. For ouabain concentrations equal to or below 2.5×10^{-8} M, [G-^{3}H]ouabain of specific activity 12 Ci/mmol was employed. For higher concentrations, 2.5×10^{-8} mmol of [^{3}H]ouabain (2.1×10^5 cpm) was diluted with appropriate concentrations of unlabeled inhibitor. The samples were incubated at 37 °C for 180 min, then filtered through Millipore HA membranes, which were then washed three times with 1 mL of histidine buffer + $MgCl_2$ (room temperature), dried, and counted in scintillation fluid (Triton X-100–toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene). Results presented are the average of duplicate determinations which differed by <10%.

Less than 5% of either (Na, K)ATPase activity or total protein, the latter determined by the Lowry method (1951), was found in the filtrates of samples prepared without ouabain.

[^{3}H]Ouabain was compared with unlabeled ouabain with respect to its ability to inhibit (Na, K)ATPase activity. No significant differences were observed in the dose–response curves over the range of concentrations tested (2.5×10^{-10} to 2.5×10^{-8} M; the concentration of ^{3}H was computed on the basis of the given specific activity).

Results

In the absence of ouabain, the specific activity of (Na, K)-ATPase measured in preparations of the wild type HeLa clone is identical with that in preparations of the ouabain-resistant mutants HI-B1 and HI-C1. Figure 1 describes the inhibition by ouabain of (Na, K)ATPase in the three preparations. Activity of the enzyme from wild type HeLa is detectably inhibited at 5×10^{-10} to 10^{-9} M and completely inhibited at 2.5×10^{-8} M ouabain. Progressive loss of (Na, K)ATPase is also observed over this range of concentrations with HI-B1 and HI-C1 preparations, but at 2.5×10^{-8} M 35 ± 3% of the initial activity remains. While the two mutants are indistinguishable

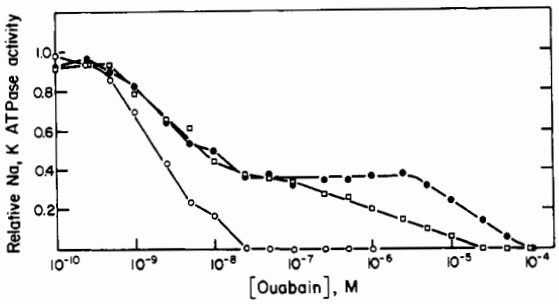

FIGURE 1: Ouabain inhibition of (Na, K)ATPase activity in preparations of wild type and mutant cells. Aliquots (5×10^{-3} EU, 40 μg of protein) of wild type HeLa (○), HI-B1 (□), and HI-C1 (●) were incubated with buffer containing 5 mM $MgCl_2$ for 10 min at 37 °C, ouabain was added to the indicated concentrations and incubation at 37 °C continued for 180 min. At this time the ATPase activity of the samples was measured as described in Experimental Procedure. The results are expressed as a fraction of the (Na, K)ATPase activity measured in samples incubated without ouabain. All results are corrected for ATPase activity measured in the absence of Na and K which in this experiment was equal to 15% of the total enzymatic activity.

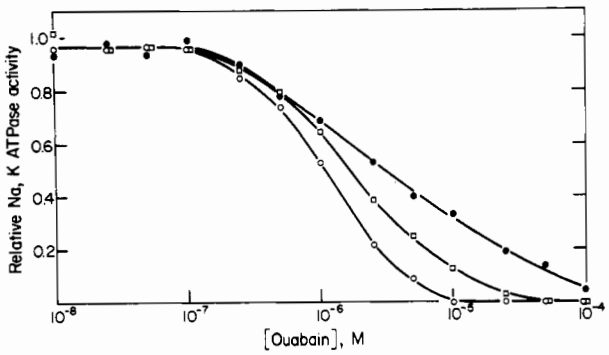

FIGURE 2: Ouabain inhibition of (Na, K)ATPase activity in the presence of NaCl. Experimental details were the same as those described in Figure 1, except 100 mM NaCl was also present during incubation of wild type HeLa (○), HI-B1 (□), and HI-C1 (●) with ouabain. Results are corrected for ATPase activity measured in the absence of Na and K which in this experiment was equal to 12% of the initial enzymatic activity.

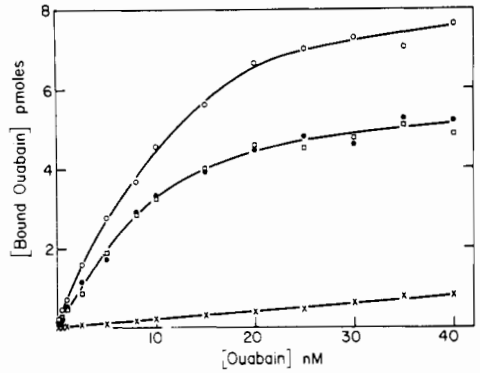

FIGURE 3: Binding of [^{3}H]ouabain to membrane preparations from wild type and mutant cells. Aliquots (5×10^{-3} EU, 40 μg of protein) of deoxycholate preparations of wild type HeLa (○), HI-B1 (□), and HI-C1 (●) were incubated with buffer containing 5 mM $MgCl_2$ for 10 min at 37 °C, [^{3}H]ouabain was added to the indicated concentrations, and incubation at 37 °C was continued for 180 min. The samples were then filtered and prepared as described in Experimental Procedure. Zero-time control values (X) were the same for the three preparations.

at less than 2.5×10^{-8} M, at higher concentrations their responses differ. HI-C1 preparations exhibit a constant level of (Na, K)ATPase activity from 2.5×10^{-8} to 2.5×10^{-6} M ouabain, which then decreases to zero at 10^{-4} M. Inhibition of HI-B1 increases steadily with increasing ouabain concentration above 2.5×10^{-8} M, but the slope describing the increase of inhibition with dose is 70 times less than that observed at concentrations below 2.5×10^{-8} M.

In the experiments shown in Figure 1, aliquots of the membrane preparations were incubated with the indicated concentrations of ouabain in buffer plus 5 mM $MgCl_2$ for 180 min prior to measurements of (Na, K)ATPase activity. At that time maximal inhibition has been achieved under these conditions; no additional inhibition was observed in either mutant or wild type preparations upon increasing the period of incubation with ouabain up to 480 min.[1]

The effects of the ligands of the (Na, K)ATPase on the sensitivity of that enzyme to ouabain have been amply documented (for a review, see Dahl and Hokin, 1974). The results presented in Figure 2 were obtained in experiments identical with those described in Figure 1, except that 100 mM NaCl was included in the buffer)as well as 5 mM $MgCl_2$) during incubation with the inhibitor. These data show that membrane preparations of wild type cells are approximately 400 times more resistant to ouabain in the presence of 100 mM NaCl; abolition of (Na, K)ATPase activity requires 10^{-5} M ouabain. In contrast, resistance of the mutant preparations is increased only 3–4-fold. In the presence of 100 mM NaCl, HI-C1 is again more resistant than HI-B1, which is in turn more resistant than HeLa wild type, but all preparations exhibit a progressive loss of enzymatic activity with increasing concentrations of ouabain. Results similar to those in Figure 2 were obtained following incubations of mutant and wild type preparations with ouabain in the presence of (1) buffer alone, and buffer plus (2) NaCl, 100 mM, (3) KCl, 10 mM, or (4) $MgCl_2$, 5 mM, and KCl, 10 mM (data not shown).

Inhibition of (Na, K)ATPase activity by cardiac glycosides has been proposed to result from a conformational change elicited in the enzyme upon binding of the inhibitor (Schwartz et al., 1972). Thus, the observed resistance to ouabain of HI-B1 and HI-C1 preparations could be explained either by reduction in the levels of ouabain bound to those preparations, or by failure to respond to the inhibitor following binding. To distinguish between these possibilities we measured the binding of [^{3}H]ouabain to membrane preparations of mutant and wild type cells, using conditions identical with those employed for the experiment described in Figure 1, i.e., incubation in buffer plus 5 mM $MgCl_2$ for 180 min. At that time maximal binding has been achieved under these conditions. As shown in Figure 3, all three preparations appear to attain saturation at 2.0–2.5 $\times 10^{-8}$ M ouabain. At saturation the number of ouabain molecules bound per mg of protein to HI-B1 and HI-C1 is 65% of that bound to wild type HeLa. These results are presented in a Scatchard plot in Figure 4. The slope of the curves describing the dependence of binding on the concentration of free inhibitor is similar for mutants and wild type.

Since (Na, K)ATPase activity of HI-B1 and HI-C1 preparations can be eliminated at elevated concentrations of oua-

[1] In order to determine whether deoxycholate, used in the preparation of the samples (Experimental Procedures), affects wild type and mutants differently, the experiments shown in Figure 1 were repeated with crude homogenates of HeLa, HI-B1, and HI-C1 prepared without detergents. Abolition of HeLa (Na, K)ATPase activity was obtained at 10^{-7} M ouabain; the responses of the mutant preparations were unchanged. In addition, no differences were observed in (Na, K)ATPase activity or ouabain inhibition of that activity between deoxycholate preparations and preparations which were subsequently treated to remove deoxycholate. The final concentration of detergent in the latter, monitored with [^{3}H]-deoxycholate, was less than 2×10^{-7} M, i.e., less than 1 molecule of deoxycholate/molecule of (Na, K)ATPase.

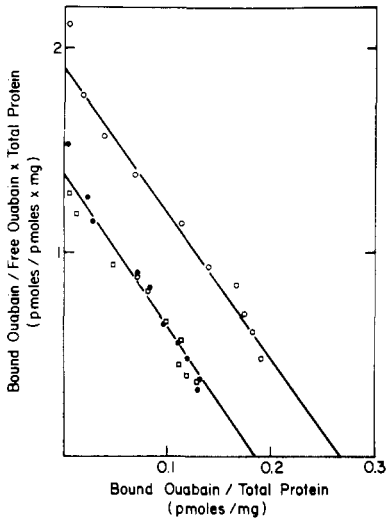

FIGURE 4: Scatchard plot of [^{3}H]ouabain binding to membrane extracts from wild type HeLa (○), HI-B1 (□), and HI-C1 (●). The results shown are from the data described in Figure 3.

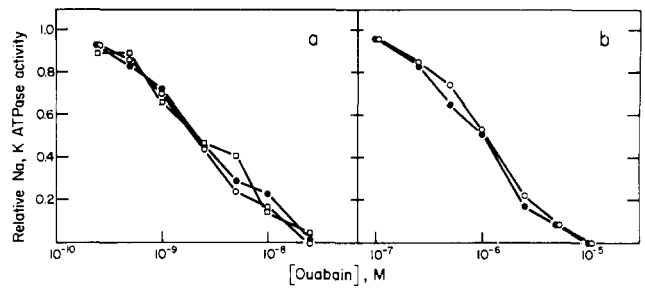

FIGURE 5: Correction of the observed ouabain inhibition of (Na, K)-ATPase activity in preparations of the mutant for the theoretical contribution of the resistant enzyme species. (a) The results shown in Figure 1 obtained for HI-B1 (□) and HI-C1 (●) at 2.5×10^{-10} to 2.5×10^{-8} M ouabain are corrected according to the formula "X = [1.5(observed relative activity − 0.33)]". The results for wild type HeLa (○) are repeated from Figure 1. (b) The results shown in Figure 2 for HI-C1 (●) are corrected as described in a, for ouabain concentration from 10^{-7} to 10^{-5} M. The results for wild type HeLa (○) are repeated from Figure 2.

bain (Figure 1), one would predict that additional binding, specific for the mutants, should occur at those concentrations. Results of binding studies performed with intact cells do suggest the existence of a second, low-affinity binding site present only on the mutants (Baker, 1976). However, we have been unable to distinguish such a site with membrane preparations of the mutants due to the high level of low-affinity binding in both mutant and wild type preparations. Included in Figure 3 is a curve describing the binding of ouabain to the preparations at zero time. This zero-time binding increases steadily with increasing ouabain concentration, and at 4×10^{-8} M contributes more than 10% of the binding observed at 180 min. At concentrations greater than 5×10^{-7} M both mutants and wild type show a sharp additional increase in the concentration of ouabain bound.

In the presence of Na, Mg plus ATP, conditions optimal for rapid binding of ouabain to the (Na, K)ATPase (Schwartz et al., 1968) it was still impossible to detect a low-affinity binding site specific for the mutants. While we found that this combination of ligands increased the affinity of both mutant and wild type preparations for the inhibitor in comparison to that measured in the presence of Mg alone (Figure 3), the number of ouabain molecules bound to the respective preparations was unchanged (data not shown).

Thus, at this point we are not certain whether the total inhibition of (Na, K)ATPase activity observed in the mutants at elevated ouabain concentrations occurs because of low-affinity binding to the enzyme itself, or as an indirect result of the large amounts of ouabain bound to other sites on the membrane preparations. Consistent with the latter possibility is the relatively small effect of Na or K on the ouabain sensitivity of the mutants (compare Figures 1 and 2), for the low affinity binding described above is not affected by the presence of these ligands. On the other hand, if the large amounts of ouabain bound to the preparations at sites other than the (Na, K)ATPase were to exert an indirect inhibitory effect on that enzyme, one might observe inhibition of other enzymes present in the preparations. We find that both 5′-nucleotidase and Mg-dependent ATPase are fully active in preparations of wild type and mutants preincubated with 10^{-4} M ouabain (data not shown), at which concentration (Na, K)ATPase activity is abolished.

The results shown in Figures 1 and 3 suggest that preparations of HI-B1 and HI-C1 contain both wild type (Na, K)-ATPase and a novel, ouabain-resistant species of the enzyme in a ratio of approximately 2:1. Under those conditions where the wild type (Na, K)ATPase is relatively ouabain sensitive (e.g., in the presence of buffer plus $MgCl_2$ alone), the activity of that species is eliminated at a concentration of the inhibitor (2.5×10^{-8} M) which is insufficient to effect any binding or inhibition of the second, resistant species. If this interpretation is valid, then correction of the results obtained in measurements of ouabain inhibition of (Na, K)ATPase activity in mutants HI-B1 and HI-C1 should result in a dose–response curve identical with that obtained for wild type HeLa. The correction is supplied by the formula: "X = [1.5 (measured relative activity − 0.33)]", where X equals the corrected relative activity of the mutants at ouabain concentrations $<2.5 \times 10^{-8}$ M. This formula simply subtracts the contribution of an (Na, K)-ATPase which is completely resistant to ouabain and which represents one-third of the total enzymatic activity in HI-B1 and HI-C1. Figure 5a illustrates the similarity of the corrected mutant curves to the empirical curve of wild type HeLa, using the results presented in Figure 1. Under all conditions tested, preparations of HI-C1 maintain one-third of their initial enzymatic activity at the minimum concentration of ouabain sufficient to abolish activity in wild type HeLa. Figure 5b compares the empirical curve for wild type with the corrected curve for HI-C1, using results obtained in the presence of $MgCl_2$ and NaCl (Figure 2), conditions where the relative sensitivity of the wild type is much reduced. The close correspondence between empirical and theoretical curves indicates that, in preparations of HI-C1, wild type (Na, K)ATPase activity is in each instance abolished at ouabain concentrations less than that required for detectable inhibition of the mutant enzyme.

The above results indicate that, although HI-B1 and HI-C1 are heterozygous with respect to the (Na, K)ATPase, the activity of the resistant enzyme can be observed in isolation by pretreating the membrane preparations with 5×10^{-8} M ouabain in the presence of 5 mM $MgCl_2$ for 180 min. We have used this procedure to compare the characteristics of ATP hydrolysis as mediated by the resistant and wild type enzyme. No significant differences were discerned (a) between mutants and wild type or (b) between mutants in the presence and absence of ouabain, with respect to the dependence of rate of hydrolysis on the concentration of ATP, Na, or Mg (Table I).

As shown in Figure 6A, ouabain-treated membrane preparations of HI-C1 exhibit a $K_{0.5}$ for potassium which is three times higher than that observed with identically treated

TABLE I: Kinetic Parameters of ATP Hydrolysis by Membranes of Wild Type HeLa, HI-B1 and HI-C1.

Sample	$K_{0.5}$ (mM) ATP[a]	Na[b]	Mg[c]
HeLa wild type	0.22	12	0.25
HI-B1	0.17	10	0.20
HI-B1 + ouabain	0.18	10	0.20
HI-C1	0.20	12	0.20
HI-C1 + ouabain	0.20	12	0.20

[a] Aliquots (7.5×10^{-3} EU, 60 μg of protein) of the membrane preparations were incubated in buffer containing 5 mM $MgCl_2$ for 10 min at 37 °C; then incubation was continued for 180 min at 37 °C in the presence or absence of 5×10^{-8} M ouabain, where indicated. At this time (Na, K)ATPase activity was assayed, as described in Experimental Procedure, at 0.05 to 3 mM ATP. Results for each concentration of ATP were corrected for activity measured, at that concentration, in the absence of Na and K. [b] Incubations and assays were performed as described above, except that activity was measured at Na concentrations ranging from 1 to 100 mM. [c] Samples were incubated in buffer containing 5 mM $MgCl_2$ at 3.75×10^{-1} EU per mL, in the presence or absence of ouabain as indicated, for 180 min at 37 °C. Aliquots were then diluted to 7.5×10^{-3} EU in assay mixtures containing $MgCl_2$ at concentrations such that the final concentration ranged from 0.10 to 10 mM. Results for each concentration of Mg were corrected for activity, at that concentration, measured in the absence of Na and K.

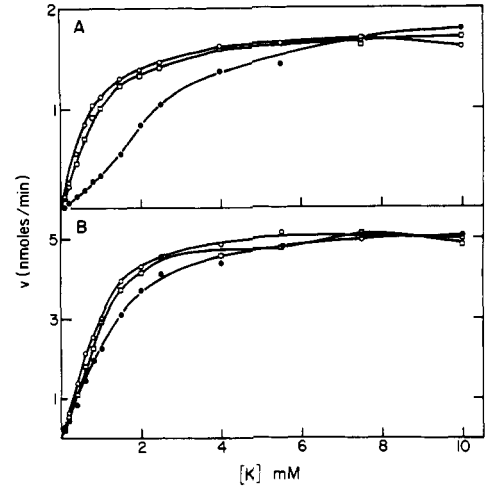

FIGURE 6: Rate of ATP hydrolysis as a function of potassium concentration. (A) Aliquots (7.5×10^{-3} EU, 60 μg of protein) of deoxycholate preparations of wild type HeLa (○), HI-B1 (□), and HI-C1 (●) were incubated with buffer containing 5 mM $MgCl_2$ for 10 min at 37 °C, then ouabain was added to final concentrations of 6×10^{-9} M (wild type) or 5×10^{-8} M (HI-B1 and HI-C1), and incubation was continued for 180 min at 37 °C. At this time (Na, K)ATPase activity was assayed using the indicated concentrations of potassium. (B) Aliquots of wild type HeLa (○), HI-B1 (□), and HI-C1 (●) were treated as described above except that ouabain was omitted from the incubation mixtures. Results are corrected for ATPase activity measured in the absence of Na and K: 0.09 nmol/min.

preparations of HI-B1, or with wild type HeLa preincubated with 6×10^{-9} M ouabain in order to reduce the activity of (Na, K)ATPase to one-third the uninhibited level. Ouabain-treated preparations of wild type HeLa, HI-B1, and HI-C1 had $K_{0.5}$'s for potassium of 0.60, 0.70, and 2.0 mM, respectively (Figure 6A). When tested without preincubation with the inhibitor, wild type HeLa, HI-B1 and HI-C1 had $K_{0.5}$'s for potassium of 0.80, 0.85, and 1.1 mM, respectively (Figure 6B).

The results presented above (Figure 1) indicate that the rate of hydrolysis by the ouabain-resistant (Na, K)ATPase of HI-C1 is not affected by increasing the inhibitor concentration from 2.5×10^{-8} to 2.5×10^{-6} M. The $K_{0.5}$ of HI-C1 for potassium also did not change over this 100-fold range of ouabain concentration.

Discussion

The results presented here demonstrate that membrane preparations of two ouabain-resistant mutants, HI-B1 and HI-C1, manifest both ouabain-resistant and ouabain-sensitive (Na, K)ATPase activity. Membranes of wild type HeLa, HI-B1, and HI-C1 are identical with respect to both total and specific activity of this enzyme. Dose-response curves describing ouabain inhibition of ATP hydrolysis indicate that one-third of the (Na, K)ATPase activity in the mutants is ouabain resistant while two-thirds of the activity exhibits a sensitivity to the inhibitor very similar to that observed with membranes of wild type HeLa. Measurements of [^{3}H]ouabain binding indicate that the membranes of the mutants bind two-thirds the number of ouabain molecules bound to membranes of wild type HeLa, over the range of concentrations which effect 0 to 100% inhibition of (Na, K)ATPase activity in preparations of wild type cells. Binding of ouabain exhibits similar concentration dependence in membranes of wild type and mutant cells.

Ouabain-resistant enzymatic activity in HI-B1 and HI-C1 was examined in isolation by pretreatment of the membranes with the appropriate concentration of the inhibitor. No significant differences were found (a) between mutants and wild type or (b) between mutants in the presence and absence of ouabain, with respect to the dependence of rate of ATP hydrolysis on the concentration of ATP, Na, or Mg. Ouabain-treated preparations of HI-C1 exhibit a $K_{0.5}$ for K which is three times greater than that measured with wild type or HI-B1 preparations, the latter tested in both the presence and absence of ouabain.

We conclude from these results that the ouabain resistance of HI-B1 and HI-C1 is due to mutation in a locus for which the aneuploid HeLa cells are triploid. HI-B1 and HI-C1 contain different mutations as evidenced by (a) the different degrees of ouabain-sensitivity observed in the two mutants both in vitro and in vivo (Baker 1976); (b) the difference in $K_{0.5}$ for K measured for ouabain-resistant (Na, K)ATPase activity in membranes of the two mutants. The simplest interpretation is that these mutations have occurred in a structural gene of the (Na, K)ATPase.

Other hypotheses may be put forth to explain the results of our kinetic studies. If one-third of the (Na, K)ATPase present in preparations of the mutants were contained in ouabain-impermeable, inside-out vesicles, then one would expect the enzymatic activity of that fraction to be ouabain resistant and the binding of ouabain to be reduced. We consider this highly unlikely, since it fails to explain differences in binding and in resistance of transport activity measured in intact cells (Baker, 1976), as well as the differences observed between ouabain-resistant enzyme activities in the two mutants. In addition, after treatment of the membrane preparations with sodium dodecyl sulfate, according to the procedure of Jørgenson (1974), we observed no alteration in the level of ouabain-resistant activity present in HI-B1 or HI-C1.

Zachowski et al. (1977) have recently reported the discovery of a protein which confers a high degree of ouabain resistance to the (Na, K)ATPase of murine plasmocytoma cells. The observed difference between mutants and wild type with respect to ouabain sensitivity might be explained if such a protein were absent from wild type HeLa but present in HI-B1 and HI-C1 at levels sufficient to affect only one-third of the total

(Na, K)ATPase. This theory does not account for the differences observed between HI-B1 and HI-C1.

A large body of evidence indicates that a molecule of (Na, K)ATPase consists of a dimer of 97 000 dalton protein (Jørgenson, 1974; Kyte, 1975; Perrone et al., 1975; Hopkins et al., 1976) plus a monomer of 50 000-dalton glycoprotein. It is the larger protein which is phosphorylated (Lane et al., 1973; Hokin et al., 1973), and in experiments involving a photoaffinity analogue of the cardiac glycoside cymarin, it is this same protein which is labeled (Ruoho and Kyte, 1974). While estimates of the number of phosphorylated sites per enzyme vary from one to two, there appears to be one ouabain binding site per $\alpha\alpha\beta$ molecule (Jørgenson, 1974; Perrone et al., 1975; Hopkins et al., 1976).

Assuming this stoichiometry, our finding that one-third of the (Na, K)ATPase activity in HI-B1 and HI-C1 is ouabain resistant would be consistent with the following possibilities: (1) it is the α monomer in only one of the two possible positions in the dimer which determines ouabain binding; HeLa cells are triploid for the α locus. Illustrating this, if α_1 and α_2 are products of the two wild type loci and α^* the product of the mutated locus, then $\alpha^* \alpha_1$, $\alpha^* \alpha_2$, and $\alpha^* \alpha^*$ would be ouabain resistant, while $\alpha_1 \alpha^*$, $\alpha_2 \alpha^*$, $\alpha_1 \alpha_1$, $\alpha_1 \alpha_2$, $\alpha_2 \alpha_1$, and $\alpha_2 \alpha_2$ would be ouabain sensitive. (2) It is the glycoprotein which determines ouabain sensitivity and HeLa cells are triploid for that locus. The experiments of Ruoho and Kyte (1974) do not eliminate the possibility that the glycoprotein contributes most of the cardiac glycoside binding surface; moreover, interpretation of their results is hindered by the fact that only 2% of the (Na, K)ATPase present was labeled by the photo-affinity analogue. (3) If HeLa cells are diploid rather than triploid for the locus responsible for ouabain binding to the enzyme, then our results would most closely coincide with a situation in which only homologous dimers of mutant proteins appear ouabain resistant. Such homologous dimers would then occur at somewhat greater than the expected frequency of 25%, unless there is an unrecognized systematic error in our measurements of both ouabain binding and inhibition of enzyme activity.

Structural analyses of purified enzyme from wild type and mutant cells should reveal the molecular explanation for our kinetic results.

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