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Glycolysis in P-glycoprotein-overexpressing human tumor cell lines

Effects of resistance-modifying agents

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We show that drugs, such as verapamil, which reverse multidrug resistance (MDR), in P-glycoprotein-overexpressing tumor cells, increased the rate of lactate production in four human MDR cell lines, but not in the parent, sensitive cell lines. The effect on glycolytic rate was maximal at a medium concentration of 2 μM verapamil. The glycolytic rate in sensitive (A2780) and MDR (2780^{AD}) cells showed the same pH dependence, but the effect of verapamil was seen only in 2780^{AD} cells at all pH values investigated (6.6, 7.4 and 8.2). A series of drugs such as nigericin, oligomycin, amiloride and monensin had similar effects in the two cells. Phorbol myristate acetate increased lactate formation in neither cell line. Verapamil induced an extra amount of ATP consumption in P-glycoprotein-expressing 2780^{AD} cells of approx. 25 pmol/s per 10^6 cells, which was estimated to be about 10% of cellular energy turnover.

Glycoprotein, P-; Glycolysis; Verapamil; Multidrug resistance

1. INTRODUCTION

Selection of tumor cells for resistance to many natural product cytotoxic agents, including anthracyclines, vinca alkaloids and colchicine, mostly results in overexpression of the *mdr1* gene [1-3], which encodes a plasma membrane protein, called P-glycoprotein. This protein appears to be homologous to bacterial transport proteins and has 12 consensus transmembrane domains and two ATP-binding sites [4]. Since active efflux of drugs like anthracyclines is thought to be responsible for the MDR phenotype [5], it has been hypothesized that P-glycoproteins might function as an energy-dependent drug efflux pump of broad specificity for toxic lipophilic agents. Drugs that reverse MDR, such as the prototype verapamil, are believed to compete with the drug efflux mechanism [6], possibly by interaction with P-glycoproteins [7]. Recently we showed that verapamil induced net ATP hydrolysis in MDR cells and suggested that interaction of verapamil with P-glycoprotein imposes a considerable cost in metabolic energy on a cell [8].

Independently it was suggested, based on experiments using the MDR human breast carcinoma cell line MCF-7/ADr^R, that an increased rate of glycolysis in MDR cells might be related to increased energy-dependent drug detoxification [9].

In particular, increased anionic glutathione transferase activity was suggested to be a determinant for increased NADPH demand coupled to increased dependency on glucose metabolism and glycolytic rate in these cells [9,10].

Here, we provide evidence that increased energy consumption by the P-glycoprotein-verapamil interaction in MDR cells is compensated for by increased glycolysis. The pH dependence and specificity of this effect are investigated.

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Abbreviations: MDR, multidrug-resistant/resistance; PMA, phorbol 12-myristate 13-acetate

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2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

Four human cancer cell lines and their MDR sublines, selected by exposure to increasing doses of doxorubicin, were used in these studies. MCF-7 and MCF-7/ADr^R human breast cancer cells [10], A2780 and 2780^{AD} human ovarian cancer cells [11], SW1573 and SW1573/500 human squamous lung cancer cells [12,13] and H-134 and H-134 AD human ovarian carcinoma cells [12,14] were routinely subcultured in Dulbecco's modification of Eagle's medium + 7.5% fetal calf serum, the resistance lines with doxorubicin until 8–12 days before experiments, as described [15]. All stocks of cells were free of mycoplasma on initiation of the experiments as tested with Hoechst stain 33258. All resistant cell lines expressed plasma membrane P-glycoprotein, as checked with monoclonal antibodies JSB-1 [12] and MRK-16 [16].

2.2. *Lactate measurements*

Exponentially growing cells were harvested, washed in ice-cold phosphate-buffered saline and suspended in incubation medium at a cell density of 2×10^6 cells/ml. The incubation medium was of the same composition as growth medium, including 4 mM glutamine and 5.6 mM D-glucose, without pH indicator and bicarbonate buffer, but with 20 mM Hepes (pH as indicated) and with 10% dialyzed fetal calf serum. Cells were incubated with drugs and extracted with perchloric acid. Supernatants were neutralized with KOH and stored at −20°C until assayed. Lactate was measured by spectrophotometric determination of NADH formation at 340 nm in the presence of excess NAD^+ and lactate dehydrogenase (Boehringer Mannheim, FRG) at pH 9.5. Because of reports that reducing equivalents derived from L-glutamine oxidation might be essential to drive lactate formation [17], in preliminary experiments the effect of omission of L-glutamine on lactate production in A2780 and 2780^{AD} was determined. No differences were observed, indicating that in both cell lines D-glucose was the major source for lactate formation.

2.3. *Oxygen consumption*

Cellular oxygen consumption was measured with a YSI 5331 oxygen probe (Yellow Springs Instrument Co.) and a Beckman model 0260 oxygen analyzer. A suspension of 2.5×10^6 cells/ml medium was pre-incubated in the measuring vial at 37°C until a stable signal was obtained, after which the vessel was sealed. O_2 consumption was measured over 10 min, after which either saline (control) or drug was injected, and O_2 consumption was recorded for 30 min. The probe was calibrated in air and in medium with sodium bisulfite added.

2.4. *Glutathione S-transferase assay*

Total glutathione *S*-transferase (EC 2.5.1.18) was assayed in cell extracts, solubilized in 0.05% Triton X-100 and thereafter sonicated [18]. Supernatants after centrifugation were incubated with 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in 0.2 M sodium phosphate buffer at pH 6.5 and 30°C. The absorbance at 340 nm was recorded for 5 min. Enzyme activity was calculated in nmol conjugate/min per mg protein. Protein was measured by the method of Bradford [19].

2.5. *Drugs*

Nigericin, monensin, phorbol myristate acetate, diltiazem, nifedipine, oligomycin, vincristine, trifluoperazine and verapamil hydrochloride were from Sigma. Daunomycin was from Specia (Paris); amiloride from Merck, Sharp & Dohme; chloroquine from Bayer, cytochalasin B from Calbiochem and Ro 11-2933/001 from Hoffmann-La Roche.

3. RESULTS

Four cell lines with proven overexpression of P-glycoprotein were assayed for the effect of verapamil on glycolysis rate. Fig.1 shows that increased basal glycolysis rates were found in two MDR lines compared to the parent cell lines (fig.1C,D) but not in the other two (fig.1A,B). Verapamil significantly increased lactate formation in all four MDR lines, but had no effect in the parent lines. In fig.2 the concentration-effect curve for the effect of verapamil in 2780^{AD} is shown. Medium concentrations of verapamil as low as 2 μM had a maximal effect, as observed previously for the effect of verapamil on depletion of cellular ATP concentrations, when respiration had been blocked [8]. Cellular oxygen consumption was 1.38 ± 0.08 and 1.31 ± 0.17 (nmol O_2/min per 10^6 cells; means ± SD for 3 experiments) for A2780 and 2780^{AD}, respectively. It was found that verapamil up to 50 μM did not affect cell respiration (not shown), indicating that there was no disturbance of ATP production from coupled oxidative phosphorylation. 10 mM NaN_3 blocked respiration completely in both cell lines.

As pH-dependent mechanisms have been implicated in reversal of MDR, suggesting that resistance-modifying agents may act by a mechanism involving their accumulation in acidic cytoplasmic structures [20,21] and since small differences in intracellular pH are known to have a large effect on aerobic glycolysis in leukocytes [22] and in tumor cells [23,24], we investigated the pH dependence of the effects of verapamil on glycolytic rate in A2780 and 2780^{AD} cells. Firstly, the results show that the response of glycolysis to changes in pH is similar in both cell lines (fig.3). As a rule the steady-state cytoplasmic pH shifts are about 50% of the external pH shift [25]. An effect of verapamil is seen at all pH values in MDR cells, but not in sensitive cells, indicating that potentially intracellular pH differences between MDR and

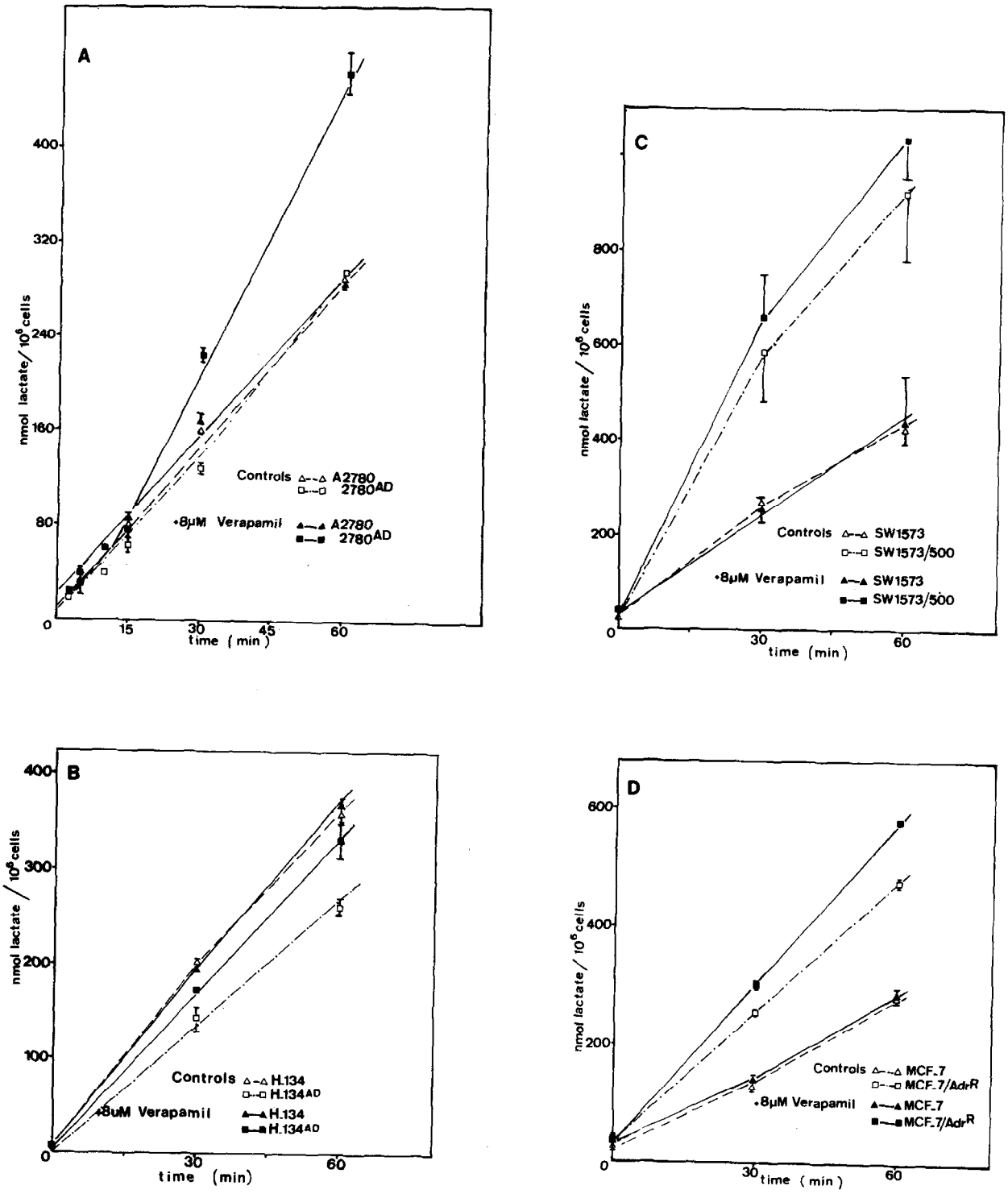

Fig.1. Verapamil effect on cellular lactate formation. For lactate measurements, cells were incubated in complete medium plus 10% dialyzed fetal calf serum. Cellular lactate was measured with a lactate dehydrogenase assay after extraction. Data are from representative experiments (duplicate samples) of 2-4 experiments per cell line.

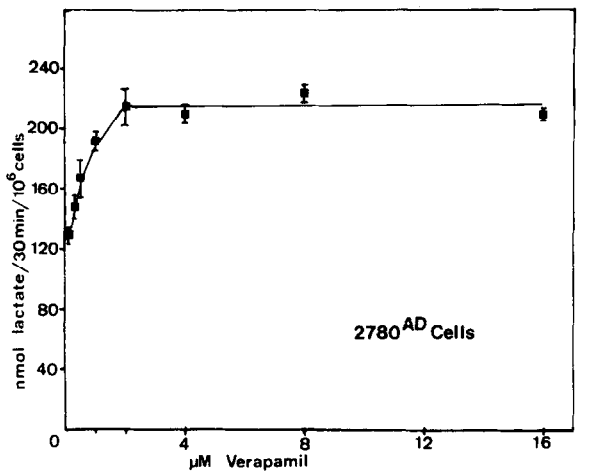

Fig.2. Verapamil effect on cellular lactate formation (30 min); concentration dependency. Data are means ± SD of 2 experiments (performed in duplicate).

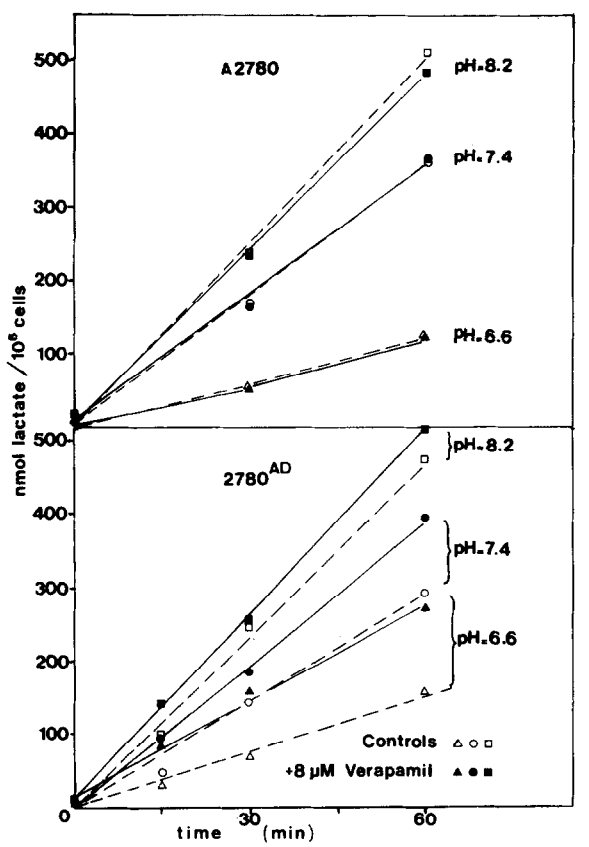

Fig.3. pH dependency of glycolysis in 2780 cells. Effects of verapamil. Data are from one of two similar experiments per cell line (duplicate samples).

sensitive cells are not likely to be responsible for the effect.

In order to establish the specificity of increases in glycolytic rate, we investigated the effects of a series of drugs with potential effects on cellular energy metabolism. Table 1 summarizes these effects. In general, three groups of compounds could be identified. One group of drugs, like verapamil, induces either an increase in lactate formation in 2780^{AD} cells exclusively or a greater increase in 2780^{AD} than in A2780 (chloroquine). A second group has similar effects in both cell lines. This group includes amiloride, an inhibitor of Na^+/H^+ exchange in various cell types [26]; monensin, an Na^+/H^+ ionophore, itself capable of increasing cytoplasmic pH [25]; nigericin, a mediator of K^+/H^+ exchange and able to uncouple oxidative phosphorylation [24]; the uncoupler oligomycin; and cytochalasin B, which lowers lactate formation in both cell lines to the same extent probably due to

Table 1

Effect of drugs on cellular lactate formation in 2780 cells

Drug	Concentration (μM)		% control	
			A2780	2780^{AD}
Verapamil	0.1		-	102 ± 1
Verapamil	0.25		-	113 ± 4[a]
Verapamil	1		-	137 ± 14[a]
Verapamil	8		102 ± 5	151 ± 21[a]
Ro 11-2933	8		104 ± 6	133 ± 15[a]
Trifluoperazine	5		105 ± 1[a]	158 ± 5[a]
Diltiazem	32		107 ± 1	147 ± 0[a]
Chloroquine	50		137 ± 6[a]	178 ± 19[a]
Amiloride	200		186 ± 54[a]	186 ± 53[a]
Oligomycin	4	(μg/ml)	149 ± 16[a]	177 ± 19[a]
Nigericin	10		162 ± 14[a]	175 ± 9[a]
Monensin	10		199 ± 23[a]	204 ± 41[a]
Cytochalasin B	20		34 ± 8[a]	34 ± 12[a]
Daunomycin	100		106 ± 9	121 ± 25
Daunomycin	200		141 ± 14[a]	150 ± 14[a]
Vincristine	100		104 ± 10	106 ± 3
PMA	0.015		104 ± 5	104 ± 7
PMA	0.15		103 ± 27	106 ± 16

[a] Statistical significant difference from control ($p < 0.05$; paired t-test)

Time of incubation was 30 min. Data are means ± SD of 3-4 experiments performed in duplicate. Control values were 4.8 ± 1.0 and 5.7 ± 0.6 nmol lactate/min per 10^6 cells ($N = 8$) for 2780^{AD} and A2780, respectively

Table 2

Glutathione transferase activity in sensitive and MDR cell lines

Cell line	Glutathione transferase activity (nmol/min per mg protein)	Ratio MDR/sensitive cells	
		Glutathione transferase	Lactate formation
A2780	61 ± 1[a]		
2780^{AD}	123 ± 3	2.0	0.8
H-134	53 ± 6		
$H\text{-}134^{AD}$	50 ± 0	0.95	0.8
SW 1573	74 ± 8		
SW 1573/500	150 ± 43	2.0	2.2
MCF-7	3 ($n=1$)		
$MCF\text{-}7/ADr^{R}$	143 ± 12	47.7	1.4

[a] Data are means ± SD of 2 experiments

inhibition of glucose uptake [27]. A third group has no effect on lactate formation in either cell line. These include daunomycin and vincristine up to 100 μM and PMA. Since detoxification mechanisms, especially increased glutathione transferase activity, have been associated with the MDR phenotype [10,28], we investigated enzyme levels in the four pairs of cells and found a correlation with neither resistance nor glycolytic rates (table 2).

4. DISCUSSION

We show that low concentrations of resistance modifiers such as verapamil cause an increase in glycolytic rate in four MDR cell lines, probably as compensation for increased ATP consumption. These four MDR lines overexpress P-glycoprotein and in at least two of them (2780^{AD}, SW1573/500) *mdr1* RNA is the only overexpressed species from the genes in the *mdr1* associated amplicon [29]. Since verapamil has been shown to bind to P-glycoprotein [7], its present effect on glycolysis is likely to be elicited via an energy-requiring interaction with P-glycoprotein, which possibly also mediates active verapamil transport. We obtained no evidence for differences in GST activities, which might account for this effect.

Another compound known to interact with P-glycoprotein is PMA. In fact, PMA causes phosphorylation of P-glycoprotein to similar levels as verapamil [30], which apparently does not require amounts of energy leading to a need for increased ATP production. Assuming 1 mol ATP/mol lactate and 5.6 mol ATP/mol O_2 consumption at complete coupling of oxidative phosphorylation [31], the calculated net ATP production in 2780^{AD} cells was 12.5 nmol ATP/min per 10^6 cells (38% contribution of glycolysis). From the difference in slope (fig.1A) it can be calculated that 8 μM verapamil caused an extra ATP turnover of 1.5 nmol/min per 10^6 cells, which is 12% of basal ATP turnover. This is a major ATP-consuming process when compared to other energy-requiring processes as estimated for Ehrlich ascites tumor cells on incubation in amino-acid-rich medium: about 30% for protein synthesis, 20% for Na^+/K^+-ATPase and approx. 10% for RNA synthesis, ATP-dependent proteolysis and Ca^{2+}-ATPase [32].

Another finding in the present study concerns the effects of drugs like chloroquine, monensin and nigericin on cellular lactate formation. Lysosomotropic amines, such as chloroquine, and proton ionophores, which are known to alkalinize acidic vesicles [33], have been shown to increase drug sensitivity of cells by increasing anthracycline accumulation [20,34,35]. Our studies suggest that the action of these drugs is not different in MDR and parent, sensitive cells and therefore not directly dependent on P-glycoprotein overexpression.

The present assay may provide a useful additional test for agents that reverse MDR. The finding that verapamil also affects MDR cells also at pH 6.6 may be of clinical importance, since such a low pH appears to be a common feature in solid tumors [36]. Further, it would be interesting to explore a possible relation between the major effects of resistance-reversing agents on cellular energy metabolism and increased cytostatic effects often observed for such drugs in MDR cells [37].

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