Induction by verapamil of a rapid increase in ATP consumption in multidrug-resistant tumor cells

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ABSTRACT

A marked increase in cellular ATP consumption was induced by verapamil in the multidrug-resistant (MDR) cell line 2780AD, but not in the drug-sensitive parental cell line A2780. A group of structurally unrelated drugs in concentrations known to reverse MDR, but not the verapamil analog tiapamil, a weak modulator of MDR, had similar effects. This effect was saturated at verapamil concentrations of about 1 μM. These data demonstrate that verapamil concentrations in MDR cells are maintained at a low level at the expense of ATP hydrolysis, and provide a first indication of the amount of metabolic energy used in this process.


Key Words: verapamil • drug transport • ATP hydrolysis • multidrug resistance

It has been suggested that in tumor cells the reversal of multidrug resistance (MDR) by verapamil and related drugs is based on competition with cytostatic agents for an energy-dependent export pump (1–3). This pump might be related to excessive production by MDR cells of transmembrane glycoproteins called P glycoproteins (4–6), which contain two presumptive ATP-binding sites on the cytoplasmic site of the protein (7–9). The demonstration that ATP binds to P glycoprotein suggests that energy supply for drug transport could be provided by ATP hydrolysis at these sites (10). In the present experiments we attempted to measure effects of verapamil, a putative candidate drug for the P glycoprotein-related drug transport, on cellular ATP use. If verapamil reverses the defective accumulation of anthracyclines or vinca alkaloids by competing for a common energy-dependent extrusion mechanism (1–3, 11–13), it should show a higher occupation of the putative drug transporter binding sites than those drugs do, and this would permit assessment of the consumption of ATP used for the binding and/or export of verapamil.

MATERIALS AND METHODS

Cell lines

The human A 2780 and 2780AD ovarian carcinoma cell lines were obtained from R. F. Ozols (National Cancer Institute, Bethesda); hamster ovarian cells AUXB, and CH5C5 were obtained from V. Ling (Ontario Cancer Institute, Toronto, Canada). Cells were cultured as described before (14, 15), the resistant cells under the continuous presence of the selecting drug until 10–14 days before experiments. All cell lines were free of mycoplasma, as tested with Hoechst stain 33258.

Drug accumulation

Cells were cultured to near confluency, harvested by brief trypsinization, washed in phosphate-buffered saline (PBS) containing Ca2+ + Mg2+ (14), and resuspended in growth medium (pH = 7.45) at cell densities of about 10^6/ml. For the determination of drug accumulation, cells were incubated with [14C]daunorubicin (DNR), [14C]doxorubicin, [3H]vincristine (VCR), or [3H]verapamil for 60 min at 37°C, pelleted, and washed in ice-cold PBS as described in ref 14. Radioactivity was determined by liquid scintillation counting and expressed as picomoles per 10^6 cells. Mean cellular volumes of A2780 and 2780AD did not differ significantly.

ATP measurements

Cells (5 × 10^6) were incubated at 37°C in medium A (growth medium) or medium B (i.e., medium A without glucose but with 10% fetal calf serum and 10 mM sodium azide). Media contained 20 mM HEPES

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buffer; osmolality was set at 305 ± 10 mosmol and pH at 7.45. During incubation, cell viability remained >97% as determined by trypan blue exclusion. Ribonucleoside mono-, di-, and triphosphates were determined in cellular trichloroacetic acid extracts with an ion exchange high-performance liquid chromatography system described elsewhere (16).

RESULTS AND DISCUSSION

Determination of the steady-state concentrations of DNR, VCR, and verapamil in 2780AD and A2780 cells showed marked differences between these cell lines as to the accumulation of the three unrelated drugs (Table 1). The most prominent feature is the low steady-state concentration in the resistant cells. For the measurement of ATP consumption an incubation medium (B) was chosen that had a limited content of glucose originating from fetal calf serum and contained the respiration blocker sodium azide. In this medium, cellular ATP suppletion was sufficiently slow to allow detection of drug-induced changes in ATP consumption. The time course of this effect is shown in Fig. 1 for 8 μM verapamil.

Because it was imperative to determine the specificity of this effect and its relation to the ability of drugs to modify the resistant phenotype, we studied two closely related structural analogs of verapamil, i.e., Ro 11-2933/001 (N-[3,4-dimethoxyphenyl]-2-[2-naphthyl]-N-methyl-m-dithiane-2-propylamine), which is a potent inhibitor of DNR efflux but a weak calcium antagonist (18), and tiapamil, a calcium antagonist that is therapeutically effective in cardiovascular conditions (19) and has a very low potency for the stimulation of drug accumulation (Table 2). Bepridil, a structurally unrelated calcium antagonist (20) that reverses MDR (14, 15), was also included in the study. The results of the key experiment (Fig. 2) show that the effective resistance-modifying agents caused a net ATP hydrolysis in the resistant 2780AD cells but not in the sensitive parent A2780 cells. Moreover, tiapamil had no effect on either cell line, which means that a calcium-antagonist effect cannot be involved. In normal growth medium (A) there were no significant effects, probably because of compensation for the increased consumption of ATP. We found corresponding effects of Ro 11-2933/001 and tiapamil in AUXB1 and CH8C5 Chinese hamster ovary cells; that is, Ro 11-2933/001, but not tiapamil, depressed ATP levels in the MDR cell line CH8C5 and not in the parent cell line AUXB1 (not shown). Apparently there is no general, unspecific effect on cellular energy metabolism.

We then performed studies to find out whether this effect was a general feature of drugs known to reverse the MDR phenotype. The data in Table 3 show that the effects were similar to those of verapamil. DNR and VCR, even in cytotoxic concentrations, had no effect on cellular ATP levels under these conditions (with respiration blocker). This would be consistent with a lower affinity for the outward transport system (24), leading to smaller effects on ATP hydrolysis compared with verapamil.

To find out whether there is a quantitative relation between the effects of verapamil on DNR accumulation and ATP hydrolysis, we used concentration-effect curves (Fig. 3). A close to maximum effect on ATP hydrolysis was seen at verapamil concentrations as low as 0.5 μM, whereas the maximum effect on DNR accumulation was reached only at concentrations above 4 μM. This finding seems to indicate that the energy-consuming action of verapamil is saturated at rather low verapamil medium concentrations. Higher concentrations of verapamil may compete more efficiently with cytostatic drugs such as DNR or vinblastine for binding to P glycoprotein (25), which would lead to increased inhibition of the efflux of the latter drugs even at a saturated carrier, because the relative concentration of drugs at P glycoprotein sites would be an important factor. The present findings are consistent with recent

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**TABLE 1. Steady-state drug concentration in 2780 cells**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, pmol/10⁶ cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A2780</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>433 ± 41</td>
</tr>
<tr>
<td>Vincristine</td>
<td>9.4 ± 0.4</td>
</tr>
<tr>
<td>Verapamil</td>
<td>30.3 ± 5.0</td>
</tr>
</tbody>
</table>

*Drugs were used at a concentration of 1 μM. Steady-state levels of drugs in these cell lines were reached within 60 min, as determined for verapamil in this study (steady state reached at 30 min), in ref 14 for daunorubicin, and in ref 17 for vincristine. *Results are means ± SD of three experiments.
reports that P glycoproteins have an ATP-binding site (7–9) and the ability to bind verapamil (25).

Verapamil and trifluoperazine enhance the phosphorylation of P glycoproteins (26–28). This increased steady-state phosphorylation is probably not related to an ATP concentration decrease of the order of magnitude we measured. Inasmuch as the binding of verapamil to membrane vesicles of resistant cells has been reported to be independent of energy input (25), the increase in ATP utilization we measured may not be related to verapamil membrane binding as such. However, a continuous efflux from the MDR cells would have to keep up with a continuous influx of a lipophilic compound such as verapamil into the cells. A strong demand for cellular energy would be associated with active pumping of these drugs. From the phase of rapid decline of the ATP level induced by 8 μM verapamil in 2780AD but not in A2780 cells (Fig. 1), a minimum use of about 150 pmol ATP/(min·10^6 cells) can be calculated. Because of compensatory ATP synthesis via glycolytic processes, this value is probably higher. Determination of verapamil steady-state concentrations

### TABLE 2. Effect of verapamil and two analogs on cellular doxorubicin concentration

<table>
<thead>
<tr>
<th>Drug, μM</th>
<th>Concentration, % A2780</th>
<th>2780AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>100*</td>
<td>100</td>
</tr>
<tr>
<td>Verapamil, 8</td>
<td>92</td>
<td>142</td>
</tr>
<tr>
<td>Ro 11-2933, 4</td>
<td>100</td>
<td>175</td>
</tr>
<tr>
<td>Tiapamil, 60</td>
<td>94</td>
<td>102</td>
</tr>
</tbody>
</table>

*For determination of doxorubicin accumulation (2 μM extracellular concentration, 60 min at 37°C), see Materials and Methods. Results are expressed as percentage of control value in four (2780AD, 100% = 53 pmol/10^6 cells) or two (A2780, 100% = 154 pmol/10^6 cells) experiments. SD was <10% in all cases.

### TABLE 3. Effect of drugs on cellular ATP levels

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration, μM</th>
<th>A2780</th>
<th>2780AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil (2)^6</td>
<td>8</td>
<td>96 ± 11</td>
<td>50 ± 9</td>
</tr>
<tr>
<td>Bepridil (21)</td>
<td>8</td>
<td>101 ± 19</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>Ro 11-2933 (18)</td>
<td>1</td>
<td>97 ± 22</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>Tiapamil (18)</td>
<td>10</td>
<td>98 ± 12</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>Trifluoperazine (2)</td>
<td>5</td>
<td>109 (n = 1)</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Nifedipine (3)</td>
<td>32</td>
<td>83 (n = 1)</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Diltiazem (22)</td>
<td>32</td>
<td>113 ± 17</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>Tamoxifen (23)</td>
<td>10</td>
<td>97 ± 9</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>20</td>
<td>123 ± 29</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>Vincristine</td>
<td>20</td>
<td>106 (n = 1)</td>
<td>89 ± 5</td>
</tr>
</tbody>
</table>

*Experiments were performed two (A2780) or three (2780AD) times; incubations were in medium B during 30 min. #Reference numbers are in parentheses. Concentrations of calcium blockers as reported to be effective in reversal of MDR (except tiapamil, which is active only at very high concentrations; see also Table 2). ^p < 0.01, paired t-test; other values do not differ significantly (P > 0.05) from those of controls (no drug), which were set at 100%.

(see Table 1) gave a value of 70 pmol/10^6 2780AD cells at 8 μM verapamil. If it is assumed that 150 pmol ATP/min is required to extrude cell-associated verapamil actively from the cell and that 1 ATP is hydrolyzed per molecule of verapamil transported, the predicted verapamil turnover time would be 0.5 min. According to Kessel (24) and our preliminary data, the efflux of verapamil is indeed very rapid (t1/2 < 1 min). The extent to which this efflux represents P glycoprotein-related drug export remains to be established, but this approximation is meant to show that an amount of ATP corresponding to the measured decrease of ATP can be reasonably expected to be consumed in active drug pumping. However, detailed data about cellular drug turnover and its effects on the cellular energy balance are needed to substantiate the stoichiometry of energy

**Figure 2.** Effect of drugs on cellular ATP levels. A2780 or 2780AD cells were incubated in medium A or B for 30 min. The effects of verapamil (vp, 8 μM), bepridil (bp, 8 μM), Ro 11-2933/001 (Ro, 10 μM), and tiapamil (tia, 10 μM) on cellular ATP levels were determined after 30 min of incubation. Values obtained in the absence of drug were set at 100%. Data are means of three or more duplicate determinations. *Significant difference from controls (P < 0.01, Student’s t test).
Figure 3. Effects of increasing concentrations of verapamil on cellular ATP levels and on DNR accumulation in 2780AD cells. Cells were incubated in medium B (30 min, 37°C). Results are expressed as percentage of ATP relative to controls after 30 min of incubation in medium B (100% = 1.4 nmol) (□) and percentage of DNR accumulated relative to controls (△). Values are means ± SD of two experiments.

consumption and drug membrane binding as well as pumping.

The present findings suggest that further analysis of the total cellular energy metabolism in MDR and sensitive cells would establish the energy demand of the membrane processes associated with the MDR phenotype.

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REFERENCES


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