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Competitive Inhibition by Verapamil of ATP-dependent High Affinity Vincristine Binding to the Plasma Membrane of Multidrug-resistant K562 Cells without Calcium Ion Involvement[1]

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ABSTRACT

Verapamil, a calcium channel blocker, can inhibit the efflux of antitumor agents from multidrug-resistant cells and reverse drug resistance. We have recently reported that the plasma membrane prepared from an adriamycin (ADM)-resistant variant (K562/ADM) of human myelogenous leukemia K562 cells showed ATP/Mg^{2+}-dependent high affinity binding of vincristine (VCR), which is closely related to the drug transport mechanism in this cell line. To clarify how calcium channel blockers inhibit the transport of antitumor agents from the resistant cells, we analyzed the effect of calcium channel blockers and Ca^{2+} ion on the VCR binding to K562/ADM plasma membrane. The ATP-dependent VCR binding was inhibited by calcium channel blockers (verapamil, nicardipine, and diltiazem), which are known to inhibit drug efflux from the resistant cells. Addition of [ethylenebis(oxyethylenenitrilo)]tetraacetic acid or high concentration of Ca^{2+} decreased the amount of VCR binding to some extent; however, a substantial amount of VCR still could bind to K562/ADM plasma membrane. The inhibitory effect of verapamil on the VCR binding was observed regardless of the Ca^{2+} concentration. Klotz plot analysis revealed that the inhibition of the VCR binding to K562/ADM plasma membrane by verapamil was competitive. Dissociation constant (K_d) of VCR and apparent inhibitory constant (K_i^{app}) of verapamil were calculated to be 0.1 ± 0.1 μM (SD) and 1 ± 1 μM, respectively. These results indicate that Ca^{2+} ion is not required for the VCR binding and that verapamil competitively inhibits the VCR binding without concerning Ca^{2+} ion. Antitumor agents (vinblastine, actinomycin D, ADM, and colchicine) and other agents known to reverse multidrug resistance (nicardipine, diltiazem, cyclosporin A, quinidine, and trifluoperazine) also inhibited the VCR binding competitively.

INTRODUCTION

The emergence of resistant tumor cells during treatment is one of the major problems in cancer chemotherapy. When tumor cells acquire resistance to *Vinca* alkaloids and anthracyclines, they tend to show cross-resistance to other antitumor agents (1–3). These multidrug-resistant tumor cells can actively transport the antitumor agents outside the cells and reduce the intracellular accumulation of the antitumor agents (4–7). A membrane glycoprotein termed P-glycoprotein is overexpressed in these cells (7–10). Evidence suggests that the P-glycoprotein functions as a transporter of antitumor agents on the plasma membrane of multidrug-resistant tumor cells (for reviews see Refs. 1, 11, and 12).

It is well known that certain calcium channel blockers such as verapamil, nicardipine, and diltiazem can inhibit the efflux of antitumor agents from resistant cells and can reverse drug resistance (13–18). Multidrug-resistant cells have higher calcium content than do sensitive cells (19). These findings suggest the significant role of Ca^{2+} in the transport mechanism of antitumor agents. However, it has been reported that there are no calcium channels on the plasma membrane of the ADM[3]-resistant variant (K562/ADM) of human myelogenous leukemia K562 cells (20). Photoactive analogue of Ca^{2+} antagonist can bind to the P-glycoprotein (21). Ca^{2+} antagonists may directly interact with P-glycoprotein and inhibit the drug efflux from the cells (21, 22). The mode of inhibition and possible involvement of Ca^{2+} in the process, however, has not been elucidated yet.

Recently, we reported that VCR bound to high affinity sites on K562/ADM plasma membrane depending on ATP and Mg^{2+}, and this binding is closely related to the mechanism of VCR transport (23). By using this ATP-dependent VCR-binding system, we report in this paper that Ca^{2+} ion does not have a critical role in the transport mechanism of VCR and that verapamil competitively inhibits the high affinity VCR binding.

MATERIALS AND METHODS

Chemicals. The sources of materials used in this work were as follows: [^{3}H]VCR (8.2 Ci/mmol) was from Amersham Japan, Ltd., Tokyo; ATP was from Sigma; bovine serum albumin (fraction V) was from Boehringer Mannheim; membrane filters (MF-membrane; pore size, 0.22 μm) were from Millipore. Cyclosporin A was provided by Sandoz Pharmaceuticals, Ltd., Tokyo. Antitumor agents and other drugs were obtained from commercial sources.

Plasma Membrane Preparation and Binding Assay. Human myelogenous leukemia K562 cells and their ADM-resistant variant cells (K562/ADM) established in our laboratory (7) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin. The procedure for isolation of plasma membrane from the cells was as described previously (23). The isolated plasma membrane preparations were stored at −80°C. Binding of [^{3}H]VCR to the plasma membrane preparation was measured by filtration methods. The detailed procedures were described previously (23). Briefly, plasma membrane preparation containing 50 μg of protein was incubated with ATP and [^{3}H]VCR at 25°C in 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, and 5 mM $MgCl_2$ in a total volume of 50 μl for 10 min, and then 4 ml of ice-cold buffer were added to stop the reaction. The plasma membrane was collected on membrane filter pretreated with 3% bovine serum albumin solution and washed with another 4 ml of ice-cold buffer. The filters were dried and radioactivity on each filter was measured.

RESULTS

ATP-dependent Binding of VCR to K562/ADM Plasma Membrane. Plasma membrane preparations from K562 and K562/ADM cells were incubated with [^{3}H]VCR in the presence or absence of ATP (Table 1). K562/ADM plasma membrane bound a large amount of VCR (230 pmol/mg protein) in the presence of 3 mM ATP, but only a marginal amount (35 pmol/mg protein) in the absence of ATP. K562 plasma membrane, on the other hand, bound a small amount of VCR with or without ATP.

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[3] The abbreviations used are: VCR, vincristine; ADM, Adriamycin.

Table 1 *ATP-dependent VCR binding to K562/ADM plasma membrane*

Plasma membrane preparations from K562/ADM or K562 cells and [^{3}H]VCR (0.5 μM) were incubated with or without ATP for 10 min. The values represent mean ± SD of triplicate determinations.

Plasma membrane	VCR bound (pmol/mg protein)	
	ATP (0 mM)	ATP (3 mM)
K562/ADM	35.4 ± 2.4	231 ± 4.7
K562	8.5 ± 0.7	11.9 ± 2.0

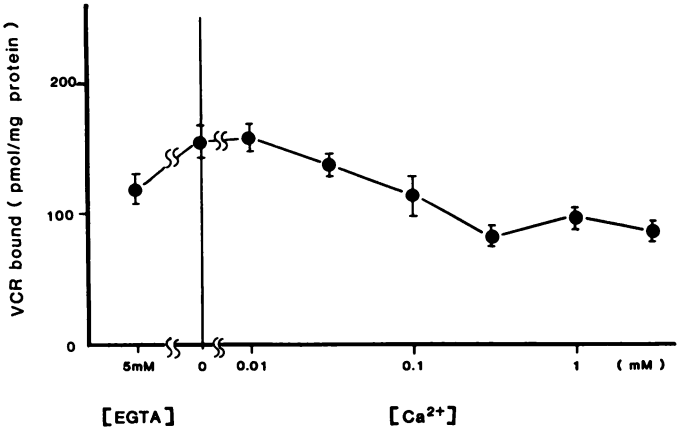

Fig. 1. VCR binding to K562/ADM plasma membrane as a function of Ca^{2+} concentration. VCR binding was measured with indicated concentration of Ca^{2+} or [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (*EGTA*). *Points*, average of triplicate determinations; *bars*, SD.

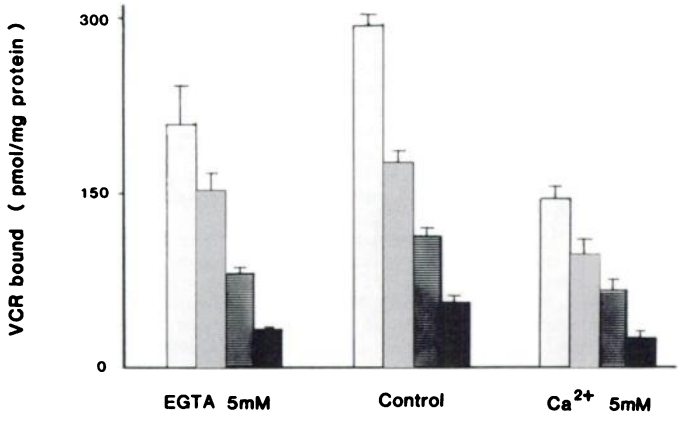

Fig. 2. Inhibition of VCR binding by verapamil at various concentrations of Ca^{2+}. VCR binding was measured with 0 μM (□); 2 μM (▨); 6 μM (▩); or 20 μM (■) verapamil at various conditions of Ca^{2+}. Data are expressed as means of triplicate determinations; *bars*, SD.

Effect of Ca^{2+} Ion and Ca^{2+} Antagonists on VCR Binding. As Ca^{2+} antagonists such as verapamil, which are known to inhibit drug efflux from resistant cells and reverse drug resistance, inhibited ATP-dependent VCR binding to K562/ADM plasma membrane (23), we tested the effect of Ca^{2+} concentration on the binding of VCR (Fig. 1). When Ca^{2+} was chelated with 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, the VCR binding was slightly suppressed. Addition of Ca^{2+} ion also suppressed the VCR binding significantly. At 3 mM Ca^{2+} the amount of VCR binding was reduced to 60%. Next, the inhibitory effect of verapamil was examined at various concentrations of Ca^{2+}. Fig. 2 shows that verapamil inhibited the VCR binding in a dose-dependent manner regardless of Ca^{2+} concentration.

In order to clarify how verapamil can inhibit the VCR binding, we further examined the VCR binding in the presence or absence of verapamil. Specific binding of VCR to K562/ADM plasma membrane in the presence or absence of 2 μM verapamil is shown in Fig. 3*a* and a double reciprocal plot (Klotz plot) of the data is shown in Fig. 3*b*. Two fitted straight lines intersect *y*-axis almost on the same point. The calculated apparent dissociation constants (K_d^{app}) were 0.1 ± 0.1 μM (SD) in the absence of verapamil and 0.4 ± 0.3 μM in the presence of 2 μM verapamil. The maximum numbers of VCR binding (B_{max}) were 220 ± 110 and 210 ± 130 pmol/mg protein with or without verapamil, respectively. Apparent inhibitory constant (K_i^{app}) of verapamil was calculated to be 1 ± 1 μM. This result clearly indicates that verapamil competitively inhibits the VCR binding. As we reported previously, ATP-independent low affinity binding of VCR to K562/ADM plasma membrane was observed (23) and verapamil also competitively inhibits the low affinity VCR binding (data not shown).

In Table 2, we summarized the apparent K_i values of the competitive inhibitors of the VCR binding, including antitumor agents (vinblastine, actinomycin D, ADM, and colchicine) to which K562/ADM cells exhibit cross-resistance, Ca^{2+} antagonists (verapamil, nicardipine, and diltiazem), and other agents (cyclosporin A, quinidine, and trifluoperazine) which are known to reverse drug resistance (24–27). Vinblastine in antitumor agents and cyclosporin A in reversing agents showed the lowest K_i^{app} values.

DISCUSSION

We described in our previous paper (23) that VCR bound to the high affinity sites on the K562/ADM plasma membrane in

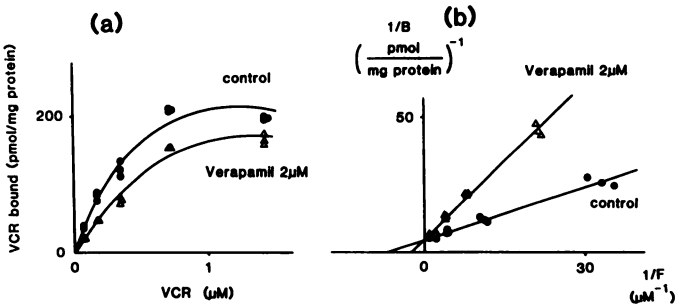

Fig. 3. Competitive inhibition of VCR binding by verapamil. *a*, specific binding of VCR in the presence or absence of 2 μM verapamil. The binding of VCR was measured as described in "Materials and Methods." Specific binding was calculated by subtraction of nonspecific binding from total binding. The nonspecific binding was measured in the presence of 100 μM VCR and was always less than 4% of the total binding. Hill coefficient for VCR binding was calculated to be 1.2 ± 0.2; *b*, double reciprocal plot (Klotz plot) of the data shown in Fig. 3*a*. The data are calculated by linear regression. The correlation coefficients were 0.96 (control) and 0.98 (2 μM verapamil). The determined binding parameters were given in the text.

Table 2 *Apparent inhibitory constants of various agents*

Apparent K_i values were determined as described in the legend of Fig. 3. Concentrations of agents used in this experiment were as follows: vinblastine, 1 μM; actinomycin D, 10 μM; ADM, 100 μM; colchicine, 100 μM; verapamil, 2 μM; nicardipine, 2 μM; diltiazem, 20 μM; cyclosporin A, 1 μM; quinidine, 2 μM; and trifluoperazine, 20 μM.

Agents	K_i^{app} (μM)
Vinblastine	0.08 ± 0.06
Actinomycin D	0.6 ± 0.4
ADM	14 ± 3
Colchicine	23 ± 9
Verapamil	1 ± 1
Nicardipine	0.7 ± 0.5
Diltiazem	8 ± 8
Cyclosporin A	0.08 ± 0.07
Quinidine	0.3 ± 0.2
Trifluoperazine	3 ± 2

the presence of ATP, while it bound to the low affinity sites in the absence of ATP. The high affinity VCR binding requires ATP hydrolysis and Mg^{2+}. The binding is inhibited by various antitumor agents to which K562/ADM cells exhibit cross-resistance and also by agents which are known to reverse drug resistance. Therefore, this ATP-dependent high affinity VCR binding apparently represents an intermediate process of VCR transports through plasma membrane. It was reported that photoactive analogues of vinblastine could bind to P-glycoprotein (28, 29). P-glycoprotein is the most plausible candidate molecule which binds and transports VCR.

Addition of a high concentration of Ca^{2+} or [ethylenebis(oxyethylenenitrilo)]tetraacetic acid decreased the amount of VCR binding to some extent; however, a substantial amount of VCR could bind to the K562/ADM plasma membrane. As described previously, ATP-dependent VCR binding requires hydrolysis of ATP (23). ATPase activity of P-glycoprotein has been reported to be reduced at high concentrations of Ca^{2+} (30, 31), and the inhibition of VCR binding by Ca^{2+} could be mediated through the inhibition of ATPase activity of P-glycoprotein by Ca^{2+}. Although Ca^{2+} ion has some influence on VCR binding, it is not required for VCR binding as is Mg^{2+} ion (23). Also, we could not detect any effects of Ca^{2+} on the dissociation rate of VCR. Therefore Ca^{2+} is not likely to be involved in the transport mechanism of VCR, and actually Ca^{2+} has no effect on the accumulation of drugs in resistant cells (data not shown).

Verapamil inhibited the VCR binding regardless of Ca^{2+} concentration. This result suggests that verapamil inhibits VCR transport by some mechanisms other than by modifying the Ca^{2+} environment. Klotz plot analysis (32) of VCR binding reveals that verapamil and other agents, which are known to inhibit drug efflux from resistant cells and reverse resistance, inhibit the VCR binding competitively. Antitumor agents to which K562/ADM cells exhibit cross-resistance also inhibit the VCR binding competitively. Such antitumor agents have been reported to be transported outside the resistant cells. Therefore, it is expected that the reversing agents listed in Table 2 are transported outside the cells just like antitumor agents. Recently we found that the accumulation of verapamil in K562/ADM cells was lower than in parental K562 cells, and this decrease was reversed by the addition of VCR or nicardipine.[4] We also found that photoactive analogue of verapamil covalently bound to the P-glycoprotein, and the binding was inhibited not only by other Ca^{2+} antagonists but also by antitumor agents such as VCR, ADM, and colchicine.[4] These findings indicate that antitumor agents such as VCR, actinomycin D, and ADM and reversing agents including verapamil bind to the P-glycoprotein competitively, and they are actively transported outside the resistant cells by the same mechanism. The reversing agents might be transported out instead of antitumor agents and inhibit the efflux of antitumor agents. The agents thus increase the accumulation of antitumor agents in resistant cells, and overcome drug resistance.

We summarized the K_i^{app} values of various agents in Table 2. Interestingly, trifluoperazine and diltiazem, having higher K_i^{app} values than verapamil and nicardipine, are less effective for overcoming drug resistance than verapamil and nicardipine (14, 15). K_i^{app} values apparently represent the affinity of each drug to the P-glycoprotein, and they would indicate the relative potency for overcoming drug resistance. Among the reversing agents, cyclosporin A shows the lowest K_i^{app} value, which suggests that cyclosporin A is highly effective in overcoming multidrug resistance. It is also of interest to note that resistance to *Vinca* alkaloids was more efficiently reversed by verapamil than resistance to ADM (15), and vinblastine has a lower K_i^{app} value than ADM. ADM might be less effectively recognized and transported outside the resistant cells by P-glycoprotein, and other mechanisms of resistance might exist in such ADM-resistant cells, which result in the incomplete overcoming of ADM resistance by verapamil.

The molecular mechanisms by which P-glycoprotein recognizes many kinds of agents with different structures are not known. Elucidation of the structure-activity relationship would be useful for the development of reversing drugs which have high affinity to the P-glycoprotein and can circumvent drug resistance, and also for the development of new antitumor agents which are not recognized by the P-glycoprotein and can kill multidrug-resistant tumor cells as effectively as sensitive cells.

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