Volume 324, number 1, 99–102 FEBS 12556 June 1993

P-glycoprotein-mediated transcellular transport of MDR-reversing agents

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Received 30 March 1993; revised version received 19 April 1993

Understanding of the interactions between P-glycoprotein and multidrug resistance (MDR) reversing agents is important in designing more effective MDR modulators. We examined transcellular transport of several MDR modulators by using a drug-sensitive epithelial cell line, LLC-PK_1, and its transformant cell line, LLC-GA5-COL300, which expresses human P-glycoprotein on the apical surface. Basal-to-apical transports of azidopine and diltiazem across the LLC-GA5-COL300 monolayer were increased and apical-to-basal transports were decreased compared to those across the LLC-PK_1 monolayer, indicating that P-glycoprotein transports azidopine and diltiazem. Movements of nitrendipine and staurosporine across the epithelial monolayer were not affected by P-glycoprotein. These results suggests that some MDR modulators exert their inhibitory effect not only by blocking the initial binding of anticancer drugs but throughout the course of the transport process.

Transcellular transport; LLC-PK_1; MDR modulator; P-glycoprotein; Multidrug resistance

1. INTRODUCTION

Multidrug resistance (MDR) is a major clinical problem in cancer chemotherapy. The MDR phenotype is often associated with high expression levels of the 170-kDa P-glycoprotein, which is encoded by the *MDR1* gene in humans [1,2]. P-glycoprotein is believed to act as an energy dependent efflux pump for MDR-related anticancer drugs. It is well known that some lipophilic agents including calcium channel blockers, protein kinase C inhibitors, and immunosuppressive agents can overcome the P-glycoprotein-associated MDR phenotype. However, interactions between P-glycoprotein and such MDR modulators have not been fully understood, although this is important in designing more effective modulators.

The calcium channel blocker verapamil and immunosuppressive agent cyclosporin A, which are among the most effective MDR modulators, have been shown to compete with *Vinca* alkaloids in binding to a high-affinity binding site (a 'common binding site') of P-glycoprotein [3,4]. Azidopine, a photoactive analogue of calcium channel blockers and also an efficient inhibitor of P-glycoprotein, was reported to inhibit *Vinca* alkaloids from binding to P-glycoprotein noncompetitively [5]. These results suggest that these agents act as antagonists for the initial binding of *Vinca* alkaloids. Moreover, it has been reported that these agents are excluded from MDR cells but not from sensitive cells, suggesting that they are substrates for P-glycoprotein [5–7]. This suggests that these modulators not only block binding of *Vinca* alkaloids to P-glycoprotein but also have inhibitory effects throughout the course of the transport process. These results also make us speculate that it is common for MDR modulators to be substrates for P-glycoprotein to transport.

The substrates for P-glycoprotein and MDR modulators are very lipophilic so that accumulation data have sometimes been difficult to interpret due to specific or nonspecific adsorption to plasma membranes and filters. We have developed a transcellular transport system [8,9] in which human P-glycoprotein was expressed specifically on the apical surface of LLC-PK_1 cells, derived from the epithelial cells of porcine kidney proximal tubule, by introducing human *MDR1* cDNA isolated from normal adrenal glands [10]. Because this transcellular transport system is useful to investigate P-glycoprotein-mediated transport without the annoyance of adsorption of lipophilic compounds, we attempted to discover if several calcium channel blockers and staurosporine, known as MDR modulators, are all transported by human P-glycoprotein.

2. EXPERIMENTAL

2.1. *Materials*

[^{3}H]Azidopine (1920 GBq/mmol, 37 MBq/ml) was obtained from Amersham. [^{3}H]Diltiazem (3185.7 GBq/mmol, 37 MBq/ml), [^{3}H]nitrendipine (2701 GBq/mmol, 37 MBq/ml), [^{3}H]staurosporine (5920 GBq/mmol, 3.7 MBq/ml), and [^{14}C]sucrose (148 MBq/mmol) were from DuPont-New England Nuclear. Microplates with bottom-fil-

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tered cups (Transwell 3414, 24.5 mm in diameter polycarbonate filter with tissue culture treatment, 3.0 μm pore size) were from Costar.

2.2. *Transcellular transport*

The host cell, LLC-PK$_1$ and the transformant cell, LLC-GA5-COL300, which expresses human P-glycoprotein on the apical surface because of the introduction of human MDR1 cDNA, were planted on bottom-filtered cups at a density of 4×10^5 and 5×10^5 cells/cm^2, respectively. LLC-GA5-COL300 was maintained with 300 ng/ml colchicine. Cells were incubated over 3 nights, and media were changed for colchicine-free medium 6 h before experiments. For measurement of transcellular transport, the medium of either the basal or the apical side of the monolayer was replaced with medium containing ^{3}H-labeled MDR modulators and 3.7 kBq/ml (24.8 μM) [^{14}C]sucrose. The cells were incubated at 37°C. Samples (25 μl) of the medium of the donor and the other side were taken at 1, 2, and 3 h, and the radioactivities measured. The paracellular fluxes were monitored by measuring the appearance of [^{14}C]sucrose in the other side. After the 3-h sampling, cells were washed with PBS twice. The filter was cut out of the cup, and cells were lysed in 1 ml of 0.3 N NaOH Then protein and modulators accumulated in the cells were measured.

3. RESULTS AND DISCUSSION

Transcellular transport of [^{3}H]azidopine, a dihydropyridine calcium channel blocker, was first examined (Fig. 1A). The amount of [^{3}H]azidopine moved across the epithelial monolayer of the host cell LLC-PK$_1$ from the apical to the basal side and that in the opposite direction were almost equal, suggesting that azidopine moved across the LLC-PK$_1$ monolayer by simple diffusion. Across the LLC-GA5-COL300 monolayer, basal-to-apical transport of azidopine was greatly increased, and apical-to-basal transport was decreased compared to those across the host cell monolayer. Because P-glycoprotein is specifically expressed on the apical surface [8], intracellular substrates will be selectively excreted on the apical side. This selective excretion should yield a overall basal-to-apical transport of the substrates. The increased basal-to-apical transport and the decreased apical-to-basal transport in Fig. 1A suggests that azidopine is a substrate for P-glycoprotein to transport. The intracellular accumulation of azidopine after 3 h was significantly decreased in LLC-GA5-COL300 cells compared to the host cells, whether azidopine was added to the apical or basal medium (Fig. 1E). This indicates, together with the decreased apical-to-basal transport across the LLC-GA5-COL300 monolayer, that azidopine was actively transported against a concentration gradient.

Nitrendipine, also a dihydropyridine calcium channel blocker, inhibits azidopine binding to P-glycoprotein

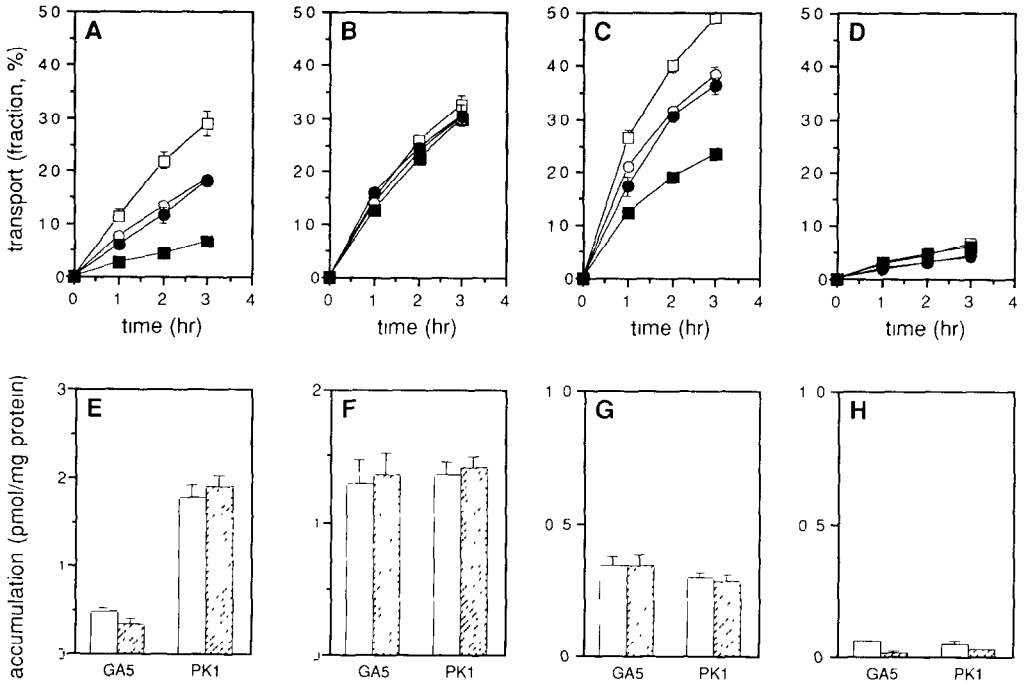

Fig. 1. Transcellular transport of lipophilic MDR modulators. (A–D) Time-dependent movement of MDR modulators through monolayers of the host cell line LLC-PK$_1$ and the P-glycoprotein-expressing transformant LLC-GA5-COL300 were measured. ^{3}H-labeled azidopine (A), nitrendipine (B), diltiazem (C), or staurosporine (D) was added in the medium of the basal side (open symbols) or of the apical side (closed symbols) of the monolayer of LLC-PK$_1$ (○,●) or LLC-GA5-COL300 (□,■). The amounts of modulators appeared in the media of the opposite side were measured after 1, 2, and 3 h and indicated as the fractions of the added amount of modulators. Each point is the average and standard error of three independent experiments. The concentrations were as follows: A, 37 kBq/ml (19.2 nM) [^{3}H]azidopine; B, 27 kBq/ml (8.6 nM) [^{3}H]nitrendipine; C, 37 kBq/ml (10.6 nM) [^{3}H]diltiazem; D, 3.7 kBq/ml (0.63 nM) [^{3}H]staurosporine (E–H) Accumulation of MDR modulators. Cells were lysed in 0.3 N NaOH after 3 h, and protein contents and accumulated MDR modulators were measured. Open bar, ^{3}H-labeled azidopine (E), nitrendipine (F), diltiazem (G), or staurosporine (H) was added to the basal side; hatched bar, ^{3}H-labeled modulators were added to the apical side. Data are shown as the averages and the standard error of three independent experiments. B and D have already been appeared in a previous report as control experiments [15].

[11], and partially overcomes the MDR phenotype in vitro (Ueda, K. et al., unpublished observation). Because these results suggest that nitrendipine directly interacts with P-glycoprotein, it is expected that nitrendipine is a substrate for P-glycoprotein. However, basal-to-apical and apical-to-basal transports of [^{3}H]nitrendipine across the LLC-GA5-COL300 monolayer were almost identical to those across the host cell monolayer (Fig. 1B), and no significant difference between intracellular accumulation in LLC-GA5-COL300 and that in LLC-PK_1 was observed (Fig. 1F). These results indicate that nitrendipine is not a substrate for P-glycoprotein-mediated transport, and is consistent with the previous report that there was no difference in binding of [^{3}H]nitrendipine between membrane vesicles prepared from the host and MDR cells [12].

Next we examined transcellular transport of a benzodiazepine calcium channel blocker, diltiazem. The basal-to-apical transport of [^{3}H]diltiazem was increased in LLC-GA5-COL300, and apical-to-basal transport was decreased compared to those in the host cells, suggesting that diltiazem was transported by P-glycoprotein (Fig. 1C). However, the amount of diltiazem accumulated in LLC-GA5-COL300 cells after 3 h was not decreased compared to that in LLC-PK_1 (Fig. 1G). This could be explained if diffusion of diltiazem across the plasma membrane was so fast (Fig. 1C circles) that P-glycoprotein expressed in LLC-GA5-COL300 could not decrease the intracellular concentration of diltiazem. The cellular accumulation of diltiazem in host cells was not high as compared to those of azidopine and nitrendipine. To measure the cellular accumulation after transcellular transport, the cells were washed with PBS, and this step required several minutes. Because diffusion of diltiazem was fast, considerable amount of diltiazem might diffused out of the cells. These results suggest that results of accumulation experiments should be interpreted carefully in deciding if a substance is a substrate for P-glycoprotein to transport.

In contrast to calcium channel blockers, a protein kinase C inhibitor, staurosporine, scarcely moved across the epithelial monolayer formed by LLC-PK_1 as well as LLC-GA5-COL300 (Fig. 1D), and quite a small amount of [^{3}H]staurosporine was accumulated in both host and transformant cells (Fig. 1H), suggesting that [^{3}H]staurosporine could not diffuse through the plasma membrane. Although staurosporine was reported to interact with P-glycoprotein directly in vitro [13], expression of P-glycoprotein had no significant effect on the movement of [^{3}H]staurosporine in LLC-GA5-COL300. Because the cytosolic concentration of staurosporine is not considered to be enough to compete with anticancer drugs, the concentration of staurosporine dissolved in the lipid phase of plasma membrane might be high enough to exert an antagonistic effect or to indirectly inhibit P-glycoprotein by modulating protein kinase C activity in vivo [14].

We have reported that the immunosuppressive agents cyclosporin A and FK506 are transported by P-glycoprotein [15]. These results and the results shown here indicate that lipophilic MDR modulators are divided into two types: ones which are transported by P-glycoprotein, and the others which are not. It is suggested that the former type of modulators, which are cyclosporin A, FK506, azidopine, diltiazem, and verapamil [7] (Saeki, T. et al., unpublished observation), exert their inhibitory effect not only by blocking the initial binding of anticancer drugs but throughout the course of the transport process, but the latter type of modulators only block the initial binding. Therefore it is conceivable that the former type of modulators are more efficient than the latter. Nitrendipine, a latter type of modulator, showed only a partial inhibitory effect (Ueda, K. et al., unpublished observation) although it strongly prevents azidopine photoaffinity labeling in vitro [11].

Because P-glycoprotein functions also as a chloride channel [16], P-glycoprotein is sometimes thought to indirectly mediate drug efflux without actually performing active drug transport, e.g. by way of changing intracellular pH. If this is the case, MDR modulators cannot be substrates for P-glycoprotein-mediated transport. By using the transcellular transport system, we clearly showed that some MDR modulators, which have been reported to bind directly to P-glycoprotein and compete with anticancer drugs, are substrates for P-glycoprotein-mediated transport. These results suggest that P-glycoprotein is directly involved in the active transport of anticancer drugs and some MDR modulators against concentration gradients, although involvement of indirect mechanisms are not be excluded. This transepithelial transport system will bring us more detailed insights for the mechanism of P-glycoprotein-mediated drug transport.

Acknowledgements. This work was supported by grants from the Ministry of Education, Science, and Culture of Japan.

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