Age-dependent Expression of P-Glycoprotein gp170 in Caco-2 Cell Monolayers

Ken-ichi Hosoya,[1] Kwang-Jin Kim,[2] and Vincent H. L. Lee[1,3]

Received January 9, 1996; accepted March 19, 1996

Purpose. To determine whether the expression and activity of the P-glycoprotein (P-GP) drug efflux pump vary with the culture age of Caco-2 cell monolayers.

Methods. Caco-2 cell monolayers were grown for 3–27 days on tissue culture-treated Transwells. P-GP efflux function was determined by measuring transmonolayer fluxes of cyclosporin A (CsA) and verapamil, while P-GP expression level was evaluated by Western blot analysis using monoclonal antibody C219.

Results. The apparent permeability coefficient (Papp) of CsA (0.5 μM) in the basolateral-to-apical (B → A) direction increased with culture age and was higher than the apical-to-basolateral (A → B) direction at all times. Net secretory Papp significantly increased from day 17 onward compared to that observed during day 3 through 13. Verapamil (100 μM) significantly inhibited CsA transport in the B → A direction from day 17 to 27, while elevating CsA transport in the A → B direction from day 6 to 27. Interestingly, the Papp of verapamil (0.5 μM) in the B → A direction was significantly higher than in the A → B direction from day 6 to 27, rendering increases in net secretory Papp of verapamil with culture age. Western analysis revealed that P-GP expression level was in the order of 4 weeks ≈ 1 week > 3 weeks > 2 weeks at equal loading of cell proteins.

Conclusions. P-GP is continuously expressed throughout the culture period, but it may not be fully functional at an early age. Caco-2 cell monolayers of day 17 to 27 appear to be a good model to evaluate the functional role of P-GP in drug efflux.

KEY WORDS: Caco-2 cell monolayer; culture age; cyclosporin A; P-glycoprotein; verapamil.

INTRODUCTION

P-glycoprotein (P-GP) is an ATP-dependent 170 KDa membrane glycoprotein associated with multidrug resistance in tumor cells (1). As such, it is responsible for reducing the intracellular accumulation of a wide variety of chemotherapeutic agents and hydrophobic compounds (2,3). P-GP is also expressed in normal tissues (4), where it possibly serves a protective role against the entry of foreign chemicals, including hydrophobic cationic drugs. In 2–3 week-old Caco-2 cell monolayers, P-GP is believed to substantially reduce the passage of cyclosporin A (CsA) (3), vinblastine (5), and peptides (e.g., $AcPhe(NMePhe)_2NH_2$) (6) through a drug efflux mechanism. As Na^+/glucose (7) and H^+/dipeptide transport processes (8) are known to progressively increase with culture age, it would be of interest to determine whether this is also the case for the expression and activity of P-GP. Thus, CsA, a very lipophilic cyclic undecapeptide known to be a substrate for P-GP (3,9), was used as the model drug to characterize the P-GP function. Verapamil was selected as the P-GP inhibitor, since it is the most commonly used for that purpose (5,9). As verapamil itself is a competitive substrate for transport by P-GP (10), its transport across Caco-2 monolayers was also evaluated as a function of culture age.

MATERIALS AND METHODS

Chemicals

[^{3}H]-Cyclosporin A (^{3}H-CsA, specific activity, 7.5 Ci/mmol) was obtained from Amersham Co. (Downers Grove, IL). D-[^{3}H]-mannitol (specific activity, 26.4 Ci/mmol), [^{3}H]-verapamil (specific activity, 66.3 Ci/mmol) and [^{3}H]-dexamethasone (specific activity, 43.9 Ci/mmol) were obtained from Dupont NEN (Boston, MA). Unlabeled cyclosporin A (CsA) was kindly provided by Sandoz Pharmaceuticals (Basel, Switzerland). Verapamil, D-mannitol and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Caco-2 cells, passage 30–50, were cultured in Dulbecco's modified Eagle's medium (DMEM, with 4.5 g/l of D-glucose) which was supplemented with 10% fetal bovine serum, nonessential amino acids (1% v/v), L-glutamine (2 mM), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Confluent cell monolayers were subcultured every 7 days by treatment with 0.5% trypsin and 0.2% EDTA. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells were plated at a density of 120,000 cells/cm^2 on tissue culture-treated Transwell inserts (12 mm diameter and 0.4 μm mean pore size polycarbonate membranes) (Costar, Cambridge, MA).

Drug Transport

Prior to each experiment, Caco-2 cell monolayers grown on Transwell inserts were washed with a pH 7.4 modified Ringer's solution containing 1 mM $CaCl_2$, 5.3 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 3.3 mM Na_2HPO_4, 137 mM NaCl, 25 mM HEPES, and 25 mM D-glucose. Following incubation at 37°C, transepithelial electrical resistance (TEER) was measured using an Epithelial VoltOhmmeter (World Precision Instruments, Sarasota, FL). CsA transport was performed following the procedure modified from that of Augustijins et al. (3), which was designed to minimize the adsorption of CsA to Transwells and maximize its recovery. Siliconized pipet tips were used in all the experiments. For consistency, the same protocol was applied to the transport studies of mannitol, dexamethasone, and verapamil each at 0.5 μM donor concentration.

Transport of ^{3}H-CsA in the Apical-to-Basolateral (A → B) Direction

Transport was initiated by adding a dosing solution containing radiolabeled CsA (0.3 μCi) to the apical compartment

[1] University of Southern California, School of Pharmacy, Department of Pharmaceutical Sciences, Los Angeles, California 90033.

[2] University of Southern California, Schools of Medicine and Engineering, Department of Medicine, Physiology and Biophysics, and Biomedical Engineering, Will Rogers Institute Pulmonary Research Center, Los Angeles, California 90033.

[3] To whom correspondence should be addressed.

0724-8741/96/0600-0885$09.50/0

(a final concentration of 0.5 μM in a total volume of 0.4 ml) of Transwell inserts bathed with 1.5 ml modified Ringer's solution in the basolateral compartment, followed by incubation at 37°C in a 5% CO_2 atmosphere and 90% relative humidity. At predetermined times, the inserts were carefully removed and transferred to new wells containing 1.5 ml fresh modified Ringer's solution. Twenty μl of 1 mM unlabeled CsA was then added to the bottom well in order to minimize radiolabeled CsA adsorption to the well. Following a 1 h incubation at 37°C, the receiver solution was removed, and placed (along with the sampling pipet tip) in a scintillation vial (first sampling). To maximize the recovery of the approximately 20% ^{3}H-CsA remaining adsorbed to the receiver well, 2 ml of modified Ringer's solution containing 10 μM cold CsA in 10% dimethylsulfoxide (DMSO) was added, incubated overnight, and transferred (along with the tip) to another scintillation vial for counting (second sampling). The amount of CsA transported (sum of both samplings) was determined by measuring the cpm in a liquid scintillation spectrometer (Beckman, Fullerton, CA). No appreciable quenching was observed.

Transport of ^{3}H-CsA in the Basolateral-to-Apical (B → A) Direction

Radiolabelled CsA (0.3 μCi) and unlabeled CsA were added separately to the basolateral compartment of the Transwell (a total concentration of 0.5 μM in a volume of 1.5 ml) and mixed with gentle agitation. Transwell inserts containing 0.4 ml modified Ringr's solution in the apical compartment were then laid down into the basolateral donor compartment to initiate transport, as described above. In time course studies, modified Ringer's solution on the apical side containing transported CsA (0.4 ml) was removed and replaced with fresh modified Ringer's solution. The apical samples (0.4 ml) were placed in scintillation vials containing 15 ml of scintillation cocktail for counting.

Transport in the Presence of Inhibitors

Verapamil (100 μM) stock solution was prepared in modified Ringer's solution containing 1% DMSO (5) and added to both the apical and basolateral compartments of the Transwell. In this experiment, the CsA dosing solution also contained verapamil at a final concentration of 100 μM.

Estimation of Apparent Permeability Coefficient

The apparent permeability coefficient (Papp) of CsA or verapamil was estimated from the slope of the linear portion of a plot of cumulative amount of the drug vs. time according to the equation: $Papp = (dQ/dt)/(A*C_0)$, where dQ/dt is the drug permeation rate (mol/sec), A is the nominal surface area of the cell monolayers (1.13 cm^2), and C_0 is the initial drug concentration (mol/ml). Two-tailed Student's t-test (for unpaired mean data) was used to determine significance of difference between mean values at $p < 0.05$.

Western Blot Analysis

Western blot was performed using the procedure modified from that of Muller et al. (11) with the C219 antihuman monoclonal antibody (Signet, Dedham, MA). Caco-2 cell monolayers grown on Transwells were lysed for 45 min in ice-cold phosphate buffer saline (PBS) containing 3% sodium dodecyl sulfate (SDS) and protease inhibitors (83 μM antipain, 73 μM pepstatin A and 0.1 mM leupeptin). The cell lysate was then centrifuged at 12,000× g for 15 min and the supernatant was used for further analysis. Total cell protein was measured by the DC protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard. Six μg of cell proteins were electrophoresed on SDS-polyacrylamide gel (8%, NOVEX, San Diego, CA) and subsequently electrotransferred to a nitrocellulose membrane (Amersham, Downers Grove, IL). Immunoblot procedure using the enhanced chemiluminescence method (ECL) was performed as per the manufacturer's protocol (Amersham, Downers Grove, IL). The level of P-GP expression was quantified in a densitometer (Bio Image, Millipore, Bedford, MA).

RESULTS

CsA Transport

The time courses of CsA transport across day 27 Caco-2 cell monolayers in both directions, both in the presence and absence of 100 μM verapamil, are shown in Fig. 1. In all instances, there was a lag time of approximately 30 min. CsA transport in the B → A direction (1.76×10^{-5} cm/s) was 7.4 times greater than that in the A → B direction (2.37×10^{-6} cm/s) ($p < 0.05$). Verapamil, at 100 μM, reduced CsA transport in the B → A direction (7.07×10^{-6} cm/s) by 40% while increasing transport in the A → B direction (4.38×10^{-6} cm/s) 1.8-times ($p < 0.05$), resulting in only a 1.6-fold difference in the Papp between the two directions.

The transport of CsA in both the A → B and B → A directions at various culture age is shown in Fig. 2A. The Papp of CsA in the B → A direction increased with culture age and was always larger than that in the A → B direction. Verapamil at 100 μM did not alter the Papp of CsA in the B → A direction during the first 13 days of culture ($p > 0.05$). At day 17, verapamil inhibited CsA transport in the B → A direction by 34% ($p < 0.05$). The corresponding value was 45% and 59% for

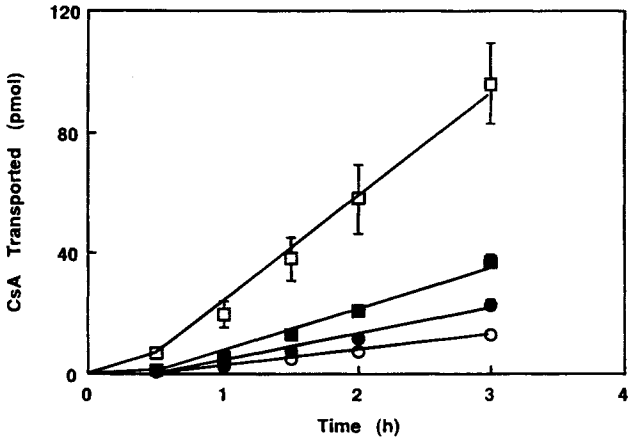

Fig. 1. Time course of CsA transport across 27 day-old Caco-2 cell monolayers both in the absence and presence of 100 μM verapamil. Each point represents mean ± s.e.m. (n = 3). Key: ○, A → B (control); □, B → A (control); ●, A → B (with verapamil); ■, B → A (with verapamil).

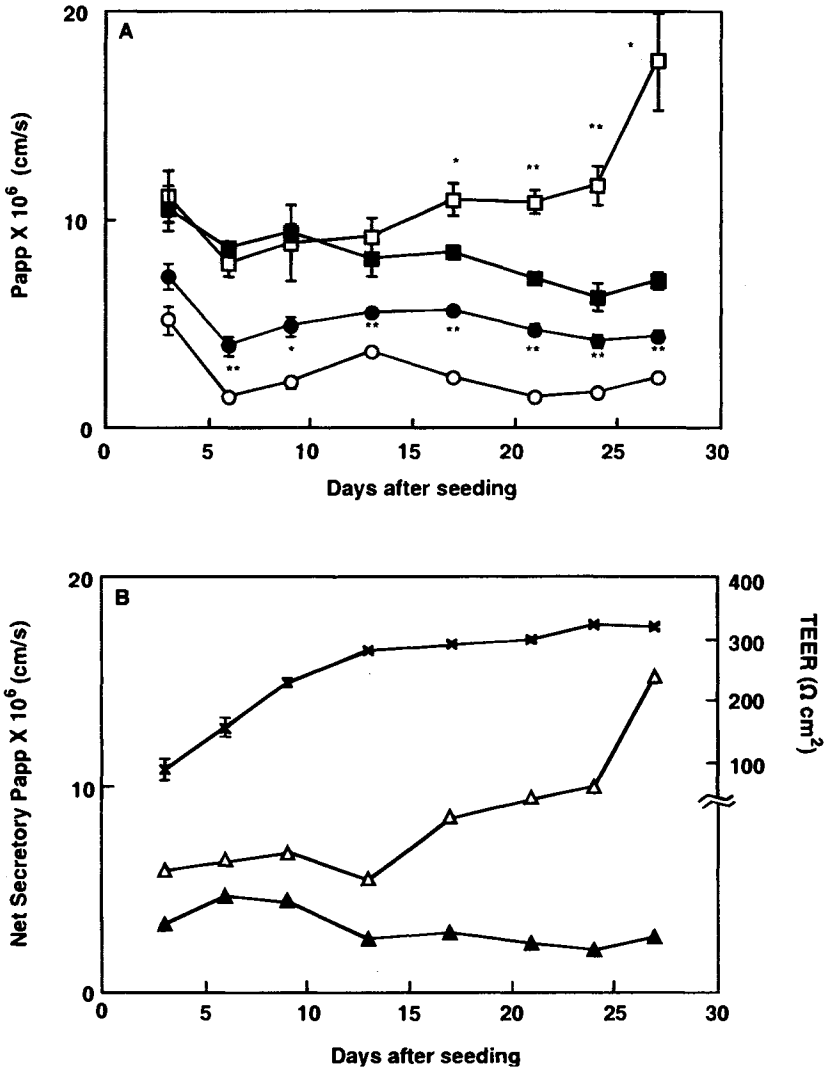

Fig. 2. Influence of culture age on CsA transport across Caco-2 cell monolayers. Transport results represent mean ± s.e.m. (n = 3–6), whereas TEER results represent mean ± s.e.m. of 24 Transwells from two separate cell preparations. Key: Plot A: ○, A → B (control); □, B → A (control); ●, A → B (with verapamil); ■, B → A (with verapamil); plot B: △, net secretory (control); ▲, net secretory (with verapamil); X, TEER. Single asterisk (*) denotes statistical significance from verapamil treatment at $p < 0.05$; double asterisks (**) denote significant difference at $p < 0.01$.

day 24 and 27, respectively. By contrast, verapamil significantly increased CsA transport in the A → B direction from day 6 through 27 ($p < 0.05$). Nevertheless, the Papp in the B → A direction remained greater than that in the A → B direction at all times. The day 3 Papp's for CsA in both directions were higher than those from day 6 to 24, probably because the cell monolayer had not yet reached confluency.

Fig. 2B shows the net secretory Papp both for CsA in the absence and presence of verapamil, where the net secretory CsA Papp was calculated by subtracting the Papp in the A → B direction from that in the B → A direction. In the absence of verapamil, the net secretory Papp was significantly elevated from day 17 onward. In the presence of verapamil, the net secretory Papp remained essentially constant throughout. TEER increased from 91.2 ± 16.1 Ωcm^2 on day 3 to 292.0 ± 4.3 Ωcm^2 on day 17 and remained constant thereafter (Fig. 2B).

To determine whether verapamil altered the Caco-2 cell monolayer permeability through paracellular and transcellular pathways (i.e., via passive diffusion), its effect on the transport of mannitol (a paracellular marker (8,12,13)) and dexamethasone (a transcellular marker (12,13)) was studied. As shown in Table 1, there was no significant difference in the Papp of mannitol between the A → B and the B → A directions on day 22 ($p > 0.1$), and verapamil did not alter mannitol transport in either direction ($p > 0.1$). While the Papp of dexamethasone in the B → A direction without verapamil was 1.2-fold higher than that in the A → B direction ($p < 0.05$), verapamil did not elicit significant changes in dexamethasone transport in either direction ($p > 0.1$). Moreover, dexamethasone transport in the A → B (Papp, 1.65 ± 0.11 × 10^{-5} cm/s) and B → A direction (1.58 ± 0.19 × 10^{-5} cm/s) at day 3 of culture was about 2 times higher than that of day 22 of culture (data not shown.)

Table 1. Effect of Verapamil on Mannitol and Dexamethasone Papp Across 22 Day-old Caco-2 Cell Monolayers

	Direction	Papp × 10^6 (cm/s) without Verapamil	Papp × 10^6 (cm/s) with Verapamil	with verapamil / without verapamil × 100 (%)
Mannitol	A → B	1.89 ± 0.58	1.91 ± 0.86	101
	B → A	3.18 ± 1.19	2.53 ± 0.24	80
Dexamethasone	A → B	7.64 ± 0.89	7.62 ± 0.96	100
	B → A	9.47 ± 0.40*	8.70 ± 0.60	92

Note: Papp entries are mean ± s.e.m. (n = 3).
*$p < 0.05$, significantly different from A → B direction.
Donor concentration of manitol and dexamethasone was 0.5 μM.

Verapamil Transport

Fig. 3 shows the effect of culture age on verapamil transport across Caco-2 cell monolayers. The net secretory verapamil Papp increased with culture age. The Papp of verapamil in the B → A direction increased with culture age and was greater than that in the A → B direction ($p < 0.01$). Specifically, the Papp of verapamil in the B → A direction on day 27 was significantly larger than those observed from day 6 through 13 ($p < 0.05$). By contrast, the Papp of verapamil in the A → B direction decreased with culture age ($p < 0.05$).

Western Blot Analysis

The expression of P-GP in Caco-2 cell monolayers from 1 to 4 weeks was determined with anti-P-GP monoclonal antibody C219 using the ECL method. A representative display of replicate determinations is shown in Fig. 4, where the band at ~170 kDa was clearly visible. Densitometric analysis revealed that the relative spot density was 100% at week 1, 81 ± 2% at week 2, 94 ± 1% at week 3, and 103 ± 1% at week 4 for 6 μg cell protein in each lane. The rank order of P-GP expression level was, therefore, 4 weeks ≈ 1 week > 3 weeks > 2 weeks.

DISCUSSION

This study has revealed that P-GP is expressed as early as day 7 of CaCo-2 cell culture on the basis of Western analysis (Fig. 4) but that it may not be fully functional until day 17 on the basis of directionality of CsA (Fig. 2) and verapamil transport (Fig. 3). CsA appears to be a better substrate for P-GP than verapamil, since the ratio of Papp's in the B → A and A → B directions is higher (7.7 vs. 1.8 at day 21). In the case of CsA, the net secretory Papp on day 6 is smaller than those observed from day 17 through 27. This may reflect a low level of P-GP activity due either to intracellular sequestration of P-GP following biosynthesis or to shedding of P-GP into the bathing medium. Indeed, Chu et al. (14) reported that soluble P-GP (180 kDa) was recovered in the culture media of fresh and viable adriamycin-resistant human ovarian carcinoma 2780 cells. At the late stage of culture (−27 days), CsA transport in the B → A direction is enhanced due to the increased level of P-GP in the apical cell membrane in conjunction with the full development of cell polarity which may be the most important factor in effecting efflux pump function.

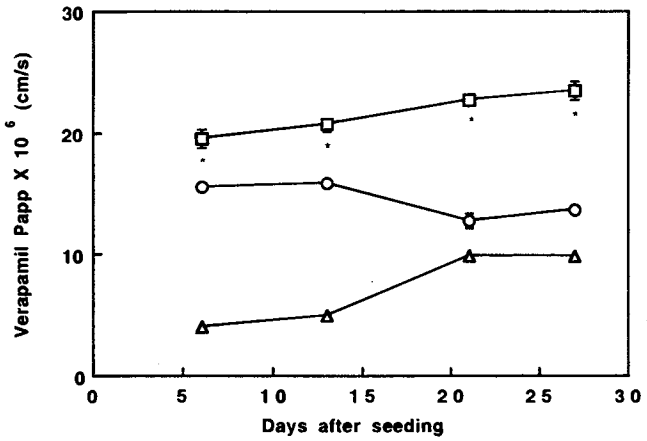

Fig. 3. Influence of culture age on verapamil transport across Caco-2 cell monolayers. Each point represents mean ± s.e.m. (n = 3–6). Key: ○, A → B; □, B → A; △, net secretory. Asterisk (*) denotes statistical significance between A → B and B → A at $p < 0.05$.

Contrary to our findings, Wils et al. (15) reported that the expression of P-GP in Caco-2 was the same from day 7 through 22 of culture according to Western blot analysis. However, these investigators used Caco-2 cells that were cultured in plastic flasks for Western blot analysis, as opposed to cells cultured on Transwells in our study. Moreover, their cell passage number (>100) was higher than ours at 30–50. Interestingly, these investigators used Transwell-grown cells for net secretion of anticancer drugs, thus making it difficult to directly correlate their Western blot results with the reported transport parameters.

Although verapamil increased CsA transport in the A → B direction as early as day 6 (Fig. 2A), it did not affect CsA transport in the B → A direction until after day 13. Conceivably, the inhibitory effect of verapamil on the low level of P-GP activity prior to day 13 may be related to changes in cell surface areas available for CsA diffusion (5,16).

In conclusion, Caco-2 cell monolayers appear to express P-GP early on in culture, but it does not become fully functional until about day 17. From the experimental point of view, Caco-2 cell monolayers grown for 17 to 27 days are well-suited for the studies of P-GP related function. Fig. 5 schematically illustrates the putative model for P-GP in Caco-2 cell monolayers based on our flux and Western blot studies.

ACKNOWLEDGMENTS

This study was supported by NIH research grants GM52812 (VHLL) and HL38658 (KJK), an unrestricted gift from the Sandoz Research Institute, and the Gavin S. Herbert Professorship (VHLL).

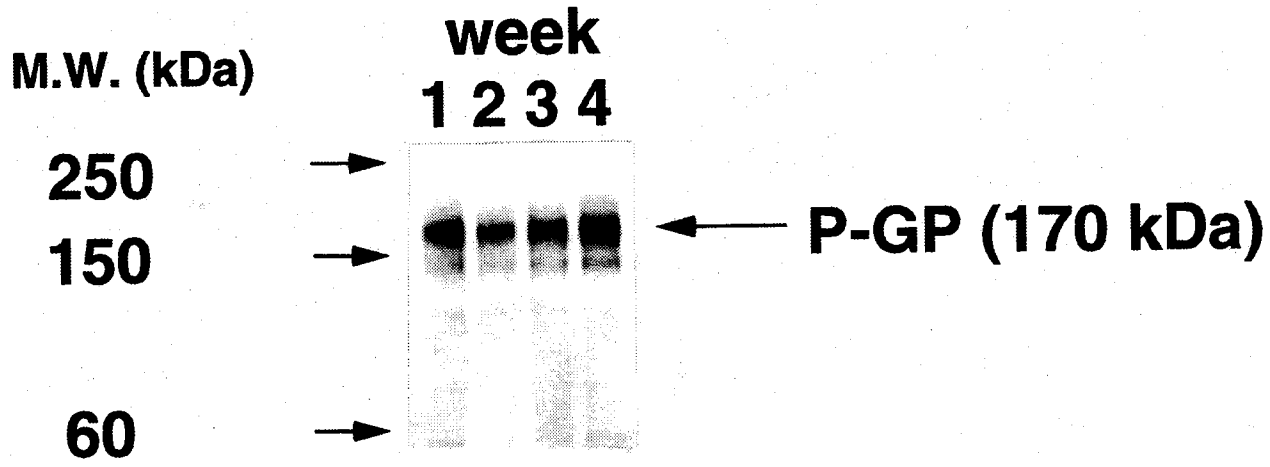

Fig. 4. Western blot analysis of P-GP expression in Caco-2 cell monolayers.

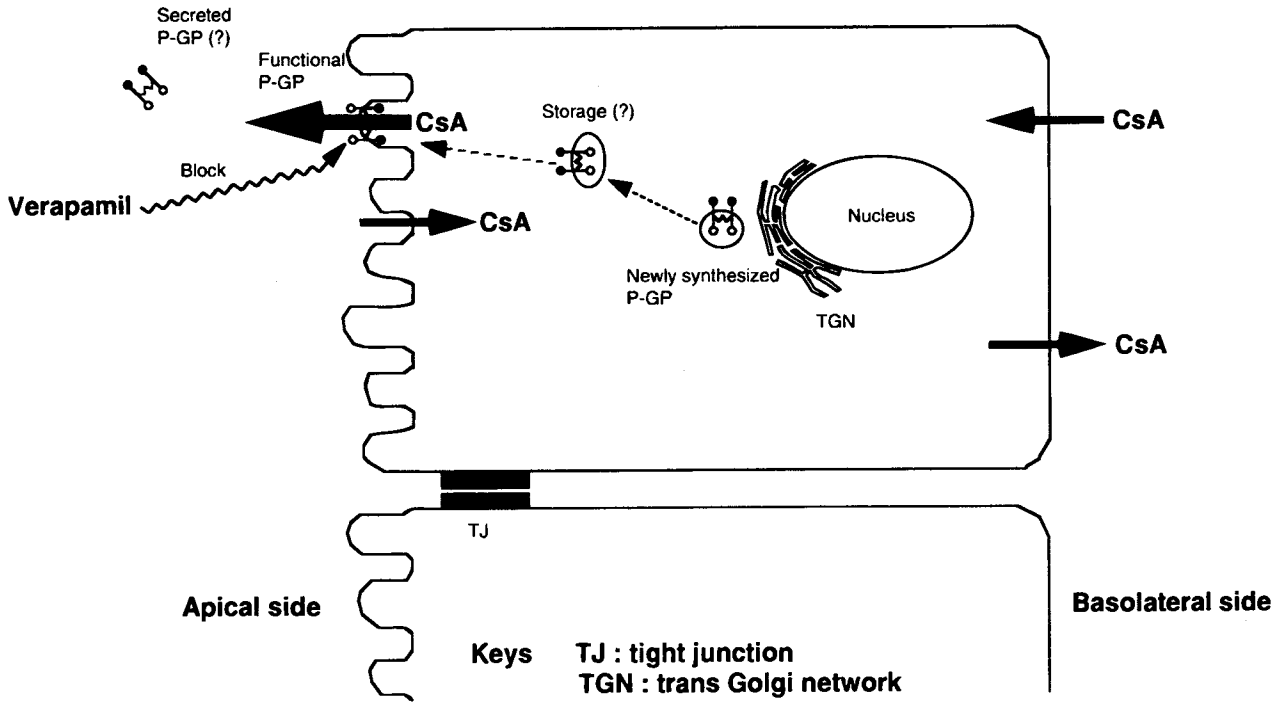

Fig. 5. Schematic illustration of the role of P-GP on CsA transport in Caco-2 cell monolayers and how it may be influenced by verapamil treatment. P-GP is synthesized and transported to the plasma membrane. At the late stage of culture, functional P-GP actively effluxes CsA, a process which is affected by verapamil.

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