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ABSORPTION ENHANCEMENT OF HYDROPHILIC COMPOUNDS BY VERAPAMIL IN CACO-2 CELL MONOLAYERS

MICHINORI SAKAI,* ARTHUR B. J. NOACH, MARGRET C. M. BLOM-ROOSEMALEN, ALBERTUS G. DE BOER† and DOUWE D. BREIMER

Leiden/Amsterdam Center for Drug Research, Division of Pharmacology, Center for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9503, NL-2300 RA Leiden, The Netherlands

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Abstract—Caco-2 monolayers were used to determine whether verapamil enhanced the transport of hydrophilic compounds across epithelial cells. Transepithelial electrical resistance (TEER) measurements, as an indicator of the opening of tight junctions, and transport experiments with fluorescein-Na (Flu) and FITC-dextran M_w 4000 (FD-4) were used to assess the effect. (±)Verapamil concentrations up to 3×10^{-4} M increased TEER dose-dependently, whereas from concentrations of 7×10^{-4} M onwards a dose-dependent drop was found. After removal of verapamil ($<10^{-3}$ M) the effects on TEER were reversible within 30 min. A second administration of verapamil after different time intervals produced a much larger effect on TEER than the first administration. The separate *R*- and *S*-enantiomers did not reveal a difference in enantiomer effect. (±)Verapamil at 7×10^{-4} M increased Flu transport about 13-fold and 26-fold after the first and second treatment in the same monolayers, respectively. Transport of FD-4 increased approximately 4-fold and 6-fold after the first and second treatment, respectively. Potential damaging effects were assessed by trypan blue exclusion (cell death) and cell detachment. No cell death occurred at verapamil concentrations of 8.5×10^{-4} M or lower, whereas cell detachment did not occur within 1 hr at all concentrations used in these experiments. At later times detachment was observed at concentrations of 7×10^{-4} M and higher. Confocal laser scanning microscopy showed that verapamil opens the paracellular route, thereby enhancing the permeability of hydrophilic compounds. However, relatively high concentrations are needed to achieve this effect and only a narrow concentration range can be used without cytotoxic effects, which limits the potential application of verapamil as an absorption enhancing agent.

Key words: verapamil; hydrophilic compounds; electrical resistance; transport enhancement; tight junctions; confocal laser scanning microscopy; permeability

The physiological regulation of intestinal epithelial paracellular permeability has long been known to involve cytoskeletal changes that act on the tight junctional structure [1–4]. This tight junction is thought to be the most important barrier in the absorption of poorly absorbed drugs, in particular hydrophilic ones. Recently, the absorption enhancing effect of various compounds has been investigated in order to improve the permeability of these hydrophilic compounds [5–7]. Several factors are known to regulate the opening of tight junctions. For example, some enhancers are likely to act via calcium chelation, suggesting that calcium ions play an essential role in the regulation of tight junctional permeability. Lowering extracellular calcium concentration has been reported to increase the permeability of the tight junction: EDTA and EGTA bind extracellular calcium ions and subsequently open tight junctions in epithelial cells [8–12]. Raising intracellular calcium concentration is another way of opening tight junctions, e.g. with the calcium ionophore A23187 [8, 13].

Thus several approaches have been used to verify the relationship between calcium and tight junctional integrity or regulation. However, it is not known whether lowering intracellular calcium concentration could influence tight junctional permeability.

(±)Verapamil‡ is a well-known drug which blocks calcium entry into the cell through voltage-sensitive calcium channels in cardiac, smooth and skeletal muscle [14–16]. Furthermore, the inhibition of calcium entry by verapamil has been observed in other cell types, e.g. in epithelial cells [17, 18]. It has been demonstrated that lowering intracellular calcium by verapamil is associated with the stimulation of water, sodium and chloride absorption into epithelial cells [19, 20]. The absorbed sodium and chloride ions are pumped into the intercellular space. As a consequence, water diffuses down its concentration gradient and might induce "solvent drag absorption" of hydrophilic drugs [21]. It has been postulated that the increment of water absorption induces an increase in the permeability

* Permanent address: Hisamitsu Pharmaceutical Co. Inc., 1-25-11 Kannondai, Tsukuba, Ibaraki 305, Japan.

† Corresponding author. Dr. A. G. de Boer, Leiden/Amsterdam Center for Drug Research, Division of Pharmacology, Center for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9503, NL-2300 RA Leiden, The Netherlands. Tel. (+31) 71 27 62 15; FAX (+31) 71 27 62 92.

‡ When verapamil is mentioned in the text, racemic (±)verapamil is meant, unless specifically stated otherwise.

of the tight junction for compounds such as creatinine, PEG, and inulin [22]. This gives rise to the hypothesis that transport of hydrophilic drugs via the paracellular route could be enhanced by calcium blocking compounds, probably by a mechanism whereby intracellular calcium concentration is lowered by verapamil at the same time as the solvent drag mechanism is stimulated.

In order to test this, Caco-2 human colonic carcinoma cells were used as a model for intestinal epithelium. Caco-2 cells grown on filters differentiate spontaneously into cells which have microvilli, desmosomes and tight junctions, and show polarity after confluency [23, 24]. Their morphological and transport characteristics seem to be good representatives of the small intestinal epithelium [11, 12, 25–27].

The effect of verapamil on paracellular permeability was assessed by measuring TEER* and the transport of Flu and FD-4 as hydrophilic model compounds. Furthermore, potential cell-damaging effects of verapamil were estimated and the transport route of the fluorescent model compounds was visualized by CLSM.

MATERIALS AND METHODS

Materials. Caco-2 cells, a human adenocarcinoma cell line, were a kind gift from Dr G. van Meer (Department of Cell Biology, Utrecht University Hospital, Utrecht, The Netherlands). (±)Verapamil was kindly provided by Dr A. A. T. M. M. Vinks (Apotheek Haagse Ziekenhuizen, The Hague, The Netherlands). *R*- and *S*-verapamil were kindly provided by Knoll BV (Amsterdam, The Netherlands).

Flu, FD-4, DMEM, non-essential amino acid solution (100×), benzylpenicillin G and streptomycin sulphate were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Fetal bovine serum was purchased from HyClone® Laboratories Inc. (Logan, UT, U.S.A.), through Greiner BV (Alphen a/d Rijn, The Netherlands).

Cell culture. Caco-2 cells were used between passages 75 and 90 throughout and were maintained routinely at 37° in 25 cm^2 cell culture flasks (Greiner, Nürtingen, F.R.G.) in DMEM (pH 7.4) supplemented with 1% non-essential amino acid solution, 10% fetal bovine serum, benzylpenicillin G (160 U/mL) and streptomycin sulphate (100 μg/mL) in an atmosphere of 95% air and 5% CO_2. The medium was changed every other day.

The cells for the experiments were grown on Transwell-COL™ collagen-treated cell culture filter inserts (Costar, Cambridge, MA, U.S.A.) with a surface area of 4.71 cm^2.

The seeding density in flasks and filters was 1×10^4 cells/cm^2. Culture medium (2.5 mL) was added to each side of the filter. The growth of the cells and the degree of confluency were checked daily by light microscopy (Olympus CK2 phase contrast inverted microscope, Olympus Optical Co., Tokyo, Japan). Confluent monolayers were used on days 21–25 after seeding, at which time the transepithelial electrical resistance of the monolayers was at a stable value of approximately 1200 Ω · cm^2. The TEER of the empty filter was approximately 20 Ω · cm^2.

Measurement of TEER. TEER of the monolayer was recorded with a four-electrode system in a custom-made measurement cup (Department of Physiology, Leiden University Medical School, Leiden, The Netherlands) filled with DMEM and kept at 37°.

The filter insert was placed between two sets of Ag/AgCl electrodes. Via a round electrode-set an a.c. of 10 μA was supplied by a 10 V, 10 Hz block pulse, generated by a PM 5231 function generator (Philips Nederland BV, Eindhoven, The Netherlands) via a 1 MΩ series resistance. The current remained constant throughout the experiments. The resulting potential difference was amplified by a Model 113 Differential Amplifier (Princeton Applied Research Corp., Princeton, NJ, U.S.A.). Signal recording was done by a PM 3350 Digital Storage Oscilloscope (Philips Nederland BV). The mean of 10 subsequent potential difference measurements was corrected for the intrinsic value of the empty filter and expressed as TEER in Ω · cm^2.

Before the start of the experiment (15 min) DMEM was changed to fresh DMEM on each side of the monolayer and immediately after the measurement at $t = 0$ DMEM was changed to DMEM containing verapamil at the apical or basolateral side. Resistance was measured periodically and calculated as a percentage of the initial ($t = 0$) value of the same monolayer.

With regard to assessment of cell recovery, both the apical and basolateral chambers were washed gently three times with fresh DMEM to remove verapamil, following an incubation period of 30 min with DMEM, with or without verapamil. Fresh DMEM was added on each side, and measurements were performed up to 300 min.

In an experiment on the enhancement of effect in relation to the time interval between successive treatments all monolayers were treated with DMEM, with or without verapamil, for 30 min as first treatment; then after 1.5 hr, 3 hr, 6 hr or 9 hr, the second verapamil treatment was performed once again for 30 min.

The integrity of the monolayer was routinely checked by light microscopy.

Transport experiments. Verapamil was dissolved in DMEM, and 2.5 mL of this solution or verapamil-free DMEM was introduced into the apical chamber. After 30 min both the apical and basolateral chambers were washed gently three times with fresh DMEM in order to wash out verapamil. Flu or FD-4 was used as a hydrophilic model marker drug and dissolved in DMEM in a final concentration of 100 μg/mL or 1 mg/mL, respectively; 2.5 mL of this solution was administered apically ($t = 0$). The basolateral chamber was bathed with 2.5 mL DMEM. Samples (200 μL) were taken at regular times from the basolateral chamber up to 180 min. The volume

* Abbreviations: TEER, transepithelial electrical resistance; Flu, fluorescein-sodium; FD-4, fluorescein isothiocyanate-labelled dextran M_w 4000; DMEM, Dulbecco's modified Eagle's Medium; CLSM, confocal laser scanning microscopy; AUC, area under the cumulative cleared volume *vs* time curve.

lost in the basolateral chamber by sampling was replaced by fresh DMEM. At $t = 180$, both the apical and basolateral chambers were washed gently three times with fresh DMEM to remove the fluorescent compound and 2.5 mL of verapamil–DMEM or verapamil-free DMEM was placed on the same monolayer apically. After 30 min ($t = 210$) both chambers were washed three times with fresh DMEM and 2.5 mL of Flu–DMEM or FD-4–DMEM was administered apically once again. Samples (200 μL) were taken at regular times from the basolateral chamber for 180 min in the same manner as in the first transport experiment. In order to obtain a concentration-independent transport parameter the clearance principle was used according to van Bree *et al.* with a custom-made APL*PLUS v8.0 program (STSC Inc., Rockville, MD, U.S.A.) [28]. For control and 3×10^{-4} M verapamil experiments, apical clearance (μL/min) could be calculated by linear regression. To compare the transport of Flu or FD-4 in control, 3×10^{-4} M verapamil and 7×10^{-4} M verapamil monolayers, the areas under the cumulative-cleared apical volume–time curves were calculated with the linear trapezoidal rule, because the cumulative-cleared apical volume did not increase linearly in monolayers treated with 7×10^{-4} M verapamil.

The integrity of the monolayers was routinely checked by light microscopy before and after the experiments.

HPLC analysis. The HPLC system consisted of a SF-400 pump (Applied Biosystems, Ramsey, NJ, U.S.A.), a WISP 710B auto-injector (Millipore-Waters, Milford, MA, U.S.A.), a stainless steel HEMA SEC BIO 300 (25 cm × 4.6 mm I.D., particle size 10 μm, Alltech, Deerfield, IL, U.S.A.) analytical column, a guard column (20 mm × 2 mm I.D., Upchurch Scientific, Oak Harbour, WA, U.S.A.) packed with HEMA SEC BIO 300 (particle size 60 μm, Alltech, Deerfield, IL, U.S.A.), a RF-530 (Shimadzu, Kyoto, Japan) or a Kontron SFM 23 (Kontron, Zürich, Switzerland) fluorescence detector operating at excitation and emission wavelengths of 488 nm and 512 nm, respectively, and a C-R3A reporting integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of 90% 75 mM NaH_2PO_4, pH 9.0/10% methanol. The flow rate was 0.5 mL/min.

FD-4 at a concentration of 12 μg/mL was used as the internal standard for Flu measurement. Vice versa, 150 ng/mL Flu was used as the internal standard for FD-4 measurement.

Trypan blue exclusion test. Following TEER measurement, both sides of the monolayers were rinsed twice with 0.01 M PBS, pH 7.4. PBS at the apical side was then replaced by trypan blue 0.1% w/v in PBS for 10 min at 37°. The solutions were then removed from both sides and monolayers checked light microscopically for the percentage of blue-stained cells by scoring in a semiquantitative manner: 0–1% (<1%), 2–5% (<5%), 6–10% (<10%), 11–30% (<30%), 31–50% (<50%), 51–80% (<80%), more than 80% (>80%) blue. Cells that excluded trypan blue were considered viable.

Cell detachment test. Cells were grown on plastic 24-well supports (Greiner, Nürtingen, Germany), surface area 2 cm^2, coated with collagen type VII (Sigma Chemical Co., St Louis, MO, U.S.A.), in 1 mL DMEM. The growth of the cells and the degree of confluency were checked daily by light microscopy. Confluent monolayers were used for the cell detachment test.

DMEM was changed to DMEM including various concentrations of verapamil, and the condition of the cells was observed microscopically. The number of holes in the monolayers was scored to estimate cell detachment. The scores were recorded at each time point. When no cell detachment was observed, the score was 0; 1–5 holes were scored as 5, 6–10 holes as 10, 11–15 holes as 15, 16–20 holes as 20, 21–25 holes as 25, and 26–30 holes as 30.

Statistical analysis. The Wilcoxon rank sum test was used to compare the data. P values <0.05 were considered to be statistically significant. Results are expressed as mean values ± SEM.

CLSM. A MRC-600 Lasersharp system (Bio-Rad Laboratories, Richmond, CA, U.S.A.) linked to a Zeiss IM 35 inverted microscope (Carl Zeiss, Oberkochen, F.R.G.) was used. All images were taken with a Zeiss Neofluar ×63, NA 1.25 oil objective. Sodium fluorescein and FD-4 were excited at a wavelength of 488 nm, whereas 514 nm served as excitation wavelength for propidium iodide. Before mounting on the microscope stage, monolayers were removed together with filter membranes from the surrounding plastic ring as follows: after aspirating apical and basolateral fluids, filters were positioned on a glass coverslip (0.15 mm thickness, 12 mm diameter, Menzel, Braunschweig, F.R.G.) lying on the bottom of an inverted film-plastic pack (30 mm diameter). A 17 mm diameter tube made from a 10 mL plastic syringe (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) was then put onto the apical filter-side, the monolayer area supported by the glass coverslip being centred within the margins of the tube. While pressing the tube downwards, the plastic support of the filter was briefly pulled in the opposite direction to remove the filter from the mounting. Through a hole which had previously been made in the centre of the plastic pack, the glass coverslip was lightly pushed together with the filter/monolayer and carefully positioned upside-down on

Table 1. Effect of various concentrations of verapamil on TEER following 2 hr treatment in Caco-2 monolayers (N = 4)

	Concentration ($\times 10^{-4}$ M)	Percentage of initial resistance ± SEM	Application side
Control	—	99.2 ± 2.7	Apical
Verapamil	1	119.2 ± 1.9*	Apical
	3	134.6 ± 1.7*	Apical
	7	7.5 ± 1.8*	Apical
	8.5	1.3 ± 0.1*	Apical
	10	0.6 ± 0.5*	Apical
	8.5	13.0 ± 2.5*†	Basolateral

* $P < 0.05$ compared to control; †$P < 0.05$ compared to apical-treated monolayers with 8.5×10^{-4} M verapamil.

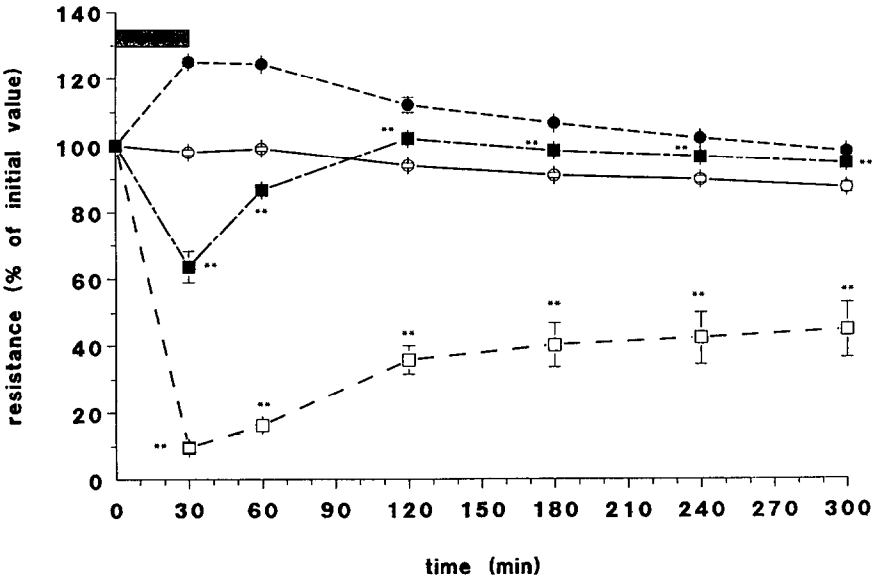

Fig. 1. Effect of various concentrations of apical verapamil on TEER. Treatment period: 0–30 min (shaded area); ○, control, N = 6 (±SEM); ●, 3×10^{-4} M verapamil, N = 2; ■, 7×10^{-4} M verapamil, N = 4; □, 1×10^{-3} M verapamil, N = 6; **P < 0.01 compared to control.

a second glass coverslip (0.15 mm thickness, 24 mm diameter, Menzel). The monolayer was thus "sandwiched" between two glass coverslips which were then mounted on the microscope stage (room temperature). This method allowed us to use an inverted microscope to observe non-fixed filter-grown cells with good optical quality, since the optical pathway did not pass the filter membrane. Images were always taken within 5–10 min after positioning the filter between the two glass coverslips. Within this time period, cells remained viable as indicated by exclusion of propidium iodide, and no optically detectable interference with paracellular permeability to sodium fluorescein or FD-4 occurred (restriction of these markers to the apical cell surface in control monolayers). To ascertain reproducibility each experiment was performed four times, thereby permitting qualitative conclusions on the distribution of the fluorescent model compounds to be drawn. Before photographing, contrast enhancement settings of the MRC 600 Lasersharp-system were applied in a way which made the pictures suitable for photographing while the message of the picture did not change compared to the original.

Before transport-visualization by CLSM, the same procedure as described under "*Transport experiments*" was followed, with the exception that in some cases, propidium iodide (1 μg/mL) was added to the medium. After the incubation period, monolayers were removed from the plastic mounting as described above for observation by CLSM.

RESULTS

The effect of verapamil on TEER

Table 1 shows the effect of verapamil on TEER at various concentrations when applied to the apical side of the monolayer for a period of 2 hr. TEER in non-treated control monolayers did not significantly change. However, in the monolayers treated with verapamil at concentrations up to 3×10^{-4} M, TEER increased slightly, whereas at concentrations of 7×10^{-4} M and higher, TEER decreased slightly but significantly.

In addition, the influence of the application side of verapamil was studied. Verapamil (8.5×10^{-4} M) applied basolaterally has a lesser effect ($P < 0.05$) than when applied apically (Table 1).

Figure 1 presents TEER vs time profiles of the monolayers during and after 30 min exposure to apical verapamil. This study was performed to evaluate the reversibility of the verapamil effect. Verapamil (7×10^{-4} and 1×10^{-3} M) gave rise to a rapid and significant ($P < 0.05$) drop in TEER within 30 min. Verapamil (3×10^{-4} M) caused an increase of TEER similar to that in the dose-dependence experiment (Table 1). After apical exposure of 3×10^{-4} M and 7×10^{-4} M verapamil the effects were completely reversible. The effect of the highest concentration (1×10^{-3} M) was not completely reversible and only returned to 51% ($P < 0.01$) of control value at 300 min.

The next series of experiments was designed to investigate the influence of repeated exposure of verapamil at 3 hr intervals. Treatment periods of 30 min were chosen because of the reversibility experiments discussed previously.

As shown in Fig. 2 three treatments of 8.5×10^{-4} M verapamil for 30 min applied on the same monolayers were studied. Successive verapamil treatments resulted in more pronounced drops in TEER; full recovery of TEER within 2.5 hr was found after each treatment.

In order to investigate the influence of the time

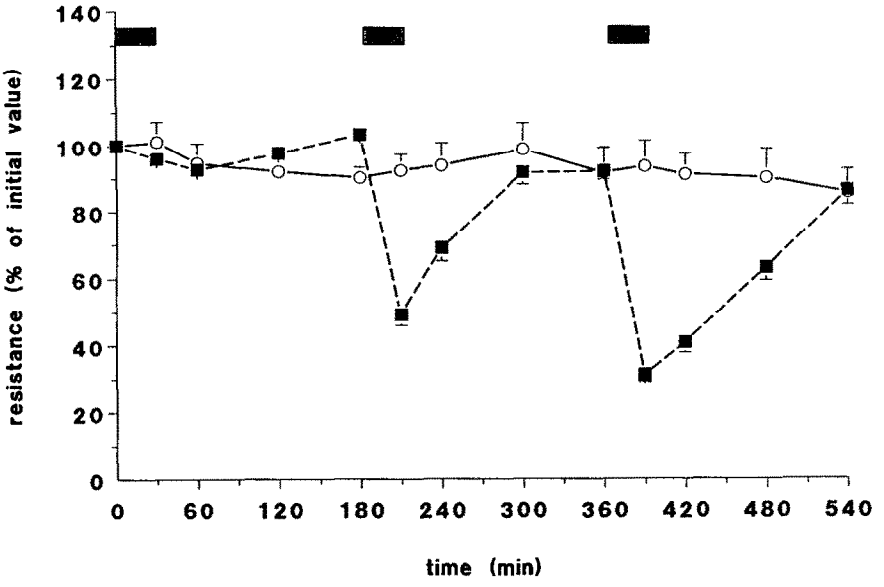

Fig. 2. Effect of the number of verapamil treatments on TEER. Monolayers were treated three times apically with 8.5×10^{-4} M verapamil (shaded areas) for 30 min; ○, control, N = 2 (±SEM); ■, verapamil, N = 3.

Table 2. Effect of two-time apical treatment with 8.5×10^{-4} M verapamil on TEER at various time intervals

Row number	Time interval (hr)	Percentage of initial resistance after 2nd treatment ± SEM*	N
1	1.5	82.9 ± 3.1	4
2	3	74.6 ± 8.4	4
3	6	18.6 ± 4.2	4
4	9	23.6 ± 7.1	4

* $P < 0.05$ when compared: row 1 to row 3, row 1 to row 4, row 2 to row 3, row 2 to row 4.

interval between the first and the second treatment of verapamil, all monolayers were first treated with 8.5×10^{-4} M verapamil for 30 min. Following specified time intervals the second treatment was performed under the same conditions as the first treatment. As shown in Table 2, the drop in TEER caused by the second verapamil application increased time-dependently with a maximal decrease being observed after 6 hr. The results of Fig. 2 and Table 2 suggest that repetitive exposure to verapamil at the monolayers causes a cumulative effect on TEER.

TEER experiments with the *R*- and *S*-verapamil enantiomers showed that stereoselectivity was not responsible for the shown effect. Here as well, 3×10^{-4} M verapamil showed a slight increase in resistance, while 8.5×10^{-4} M verapamil showed a tendency for a drop in resistance, which was nonetheless statistically not significant (Fig. 3).

The effect of verapamil on hydrophilic drug transport

Flu and FD-4, at a concentration of 100 μg/mL and 1 mg/mL, respectively, were applied to the apical side of the cells. At these concentrations neither compound influenced TEER over a period of 6 hr compared to control (data not shown). The transport experiments were carried out after 30 min verapamil exposure and repeated at 180 min (directly following the washing of the monolayers).

Figure 4 shows the results of the transport experiments with Flu. Data are presented as cumulative-cleared apical volume (V_i) vs time, i.e. the slope indicating the transport-clearance of Flu. In control monolayers, cumulative-cleared apical volumes increased linearly with time (transport-clearance values are shown in Table 3).

In monolayers treated with 3×10^{-4} M verapamil (which increased TEER as shown in Fig. 1), the cumulatively cleared apical volumes also increased linearly, but were not significantly different from those observed in control monolayers.

Verapamil treatment (7×10^{-4} M) resulted in a considerable increase in Flu transport, but this was not linear with time. Therefore, the areas under cumulatively cleared volume vs time curve (AUC) were calculated for all experiments in order to compare overall transport differences. AUC of Flu transport in 7×10^{-4} M verapamil-treated monolayers increased significantly: approx. 13-fold ($P < 0.01$) in the first experiment and approx. 26-fold ($P < 0.01$) in the second (Table 3). These results were in fairly good agreement with those observed in the TEER experiments (Fig. 2).

Similar experiments were carried out with FD-4 as a hydrophilic and relatively large M_w model compound (Fig. 5). FD-4 transport in non-treated

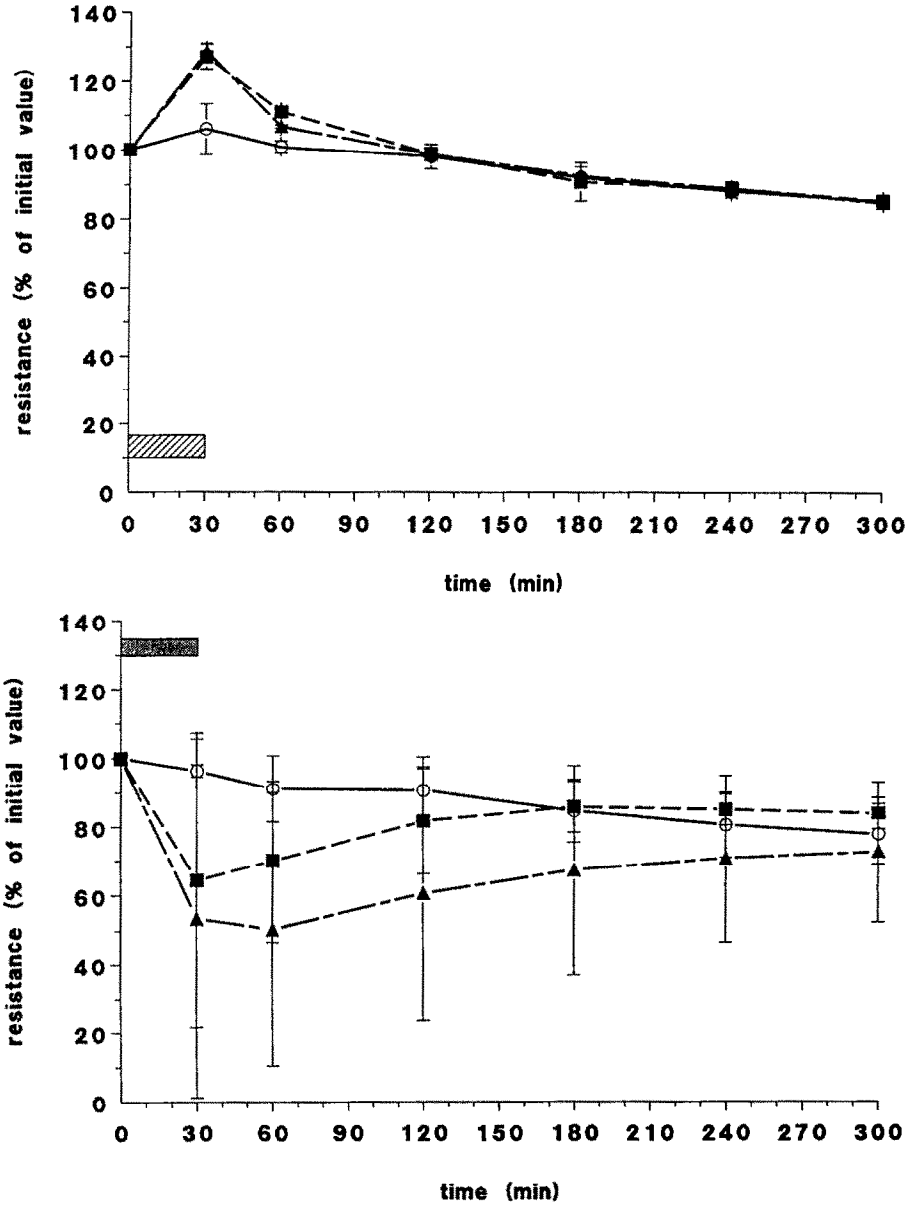

Fig. 3. Effect of various concentrations of verapamil enantiomers on TEER. Verapamil was applied to the apical side for 30 min (shaded areas). Upper panel: 3.5×10^{-4} M (N = 2); lower panel 8.5×10^{-4} M (N = 4); ○, control; ■, *R*-verapamil; ▲, *S*-verapamil.

monolayers increased linearly with time. Calculated clearance values, not significantly different for the two subsequent experiments, are given in Table 3. The AUC in 7×10^{-4} M verapamil-treated monolayers increased substantially, approx. 4-fold and 6-fold in the first and second transport experiments, respectively.

Trypan blue exclusion test

Following the TEER experiments shown in Fig. 2 and Table 2, a trypan blue exclusion test was performed to assess the viability of the monolayers (Table 4). The cells in non-treated control monolayers were virtually unstained (<1%). Following three treatments with 8.5×10^{-4} M verapamil (Fig. 2, t = 540 min), fewer than 5% of the cells were stained. In addition, two consecutive treatments with 8.5×10^{-4} M verapamil (Table 2, t = 570 min) also resulted in fewer than 5% of the cells being stained. No cell detachment could be seen microscopically following verapamil treatments and the trypan blue exclusion test.

Cell detachment test

Various concentrations of verapamil in the range of 1×10^{-4} M to 1×10^{-3} M were used to estimate

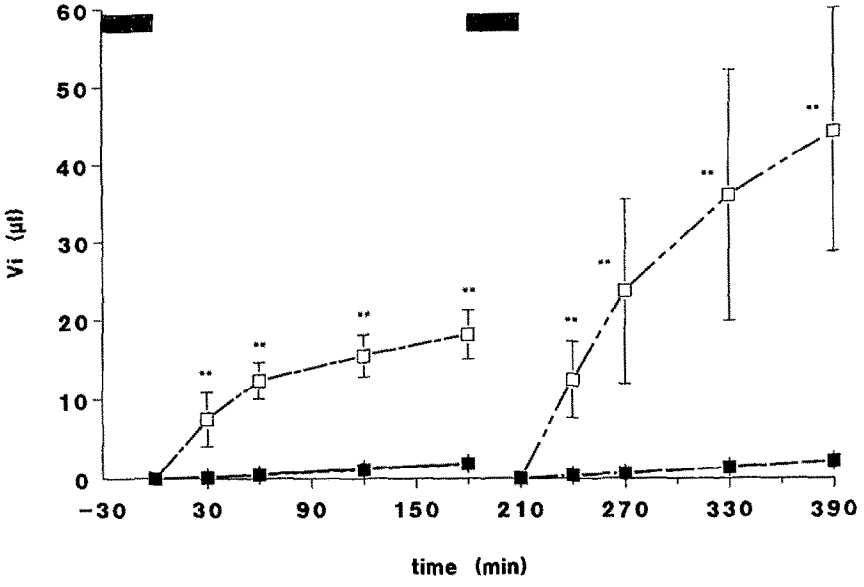

Fig. 4. Effect of verapamil on the transport of Flu across Caco-2 monolayers. Verapamil was applied to the apical side for 30 min (shaded areas); ○, control, N = 6 (±SEM); ■, 3×10^{-4} M verapamil, N = 3 (overlaps with control for all time points); □, 7×10^{-4} M verapamil, N = 4; **P < 0.01 compared to control; V_t, cumulative cleared volume of the donor chamber.

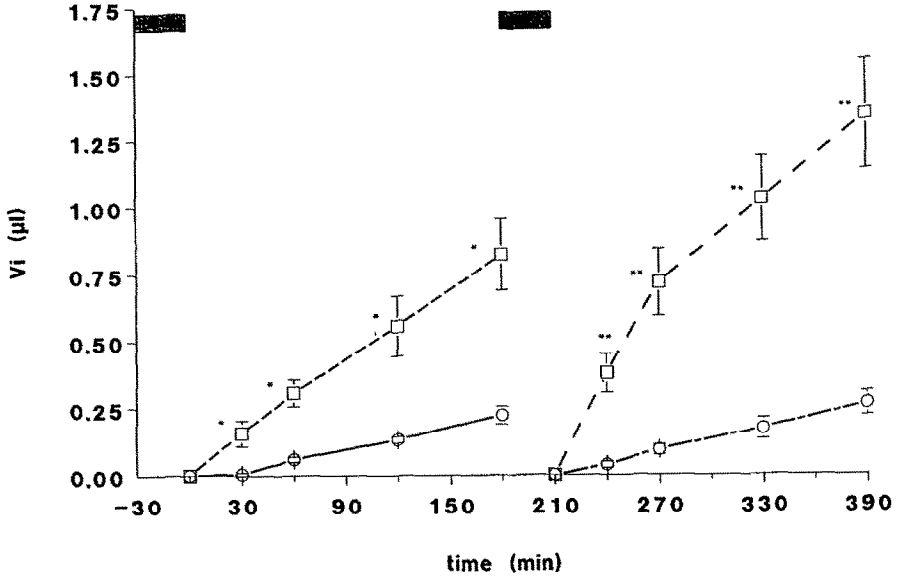

Fig. 5. Effect of verapamil on the transport of FD-4 across Caco-2 monolayers. Verapamil was applied to the apical side for 30 min (shaded areas); ○, control, N = 5 (±SEM); □, 7×10^{-4} M verapamil, N = 5; *P < 0.05, **P < 0.01 compared to control.

cell detachment by means of light microscopy. The number of holes in the monolayers was counted at regular time intervals; the results are shown in Fig. 6.

When 1×10^{-4} M to 3×10^{-4} M verapamil was applied continuously to the cell monolayers, no cell detachment could be seen for 300 min. At 7×10^{-4} M concentration cell detachment was observed after 120 min, whereas 8.5×10^{-4} M and higher concentrations of verapamil caused cell detachment as early as after 1 hr.

CLSM

Figure 7 shows the result of incubation of a monolayer with Flu when no verapamil was present. It is clear that in this case (i.e. without verapamil) the fluorescent marker compound is restricted to the apical side of the monolayer. When verapamil is

Table 3. Effect of verapamil on the amount of Flu and FD-4 transported expressed as the clearance or the area under the cumulative cleared volume *vs* time graph (N = 4)

Treatment		Flu		FD-4	
		Clearance (μL/min)	AUC (μL·min)	Clearance (μL/min)	AUC (μL·min)
Control	1st†	0.0119 ± 0.0010	178.9 ± 19.1	0.0013 ± 0.0002	17.5 ± 1.8
	2nd†	0.0126 ± 0.0025	191.1 ± 37.4	0.0015 ± 0.0003	23.5 ± 4.8
Verapamil	1st	0.0107 ± 0.0006	150.5 ± 6.9		
3×10^{-4} M	2nd	0.0119 ± 0.0009	212.4 ± 38.8		
Verapamil	1st	—	2270.9 ± 371.3**	—	76.7 ± 13.4*
7×10^{-4} M	2nd	—	4945.3 ± 2095.7**	—	145.8 ± 21.8**

* $P < 0.05$ compared to control; **$P < 0.01$ compared to control.
† 1st and 2nd transport experiment.

Table 4. Results of the trypan blue exclusion test*

Concentration verapamil ($\times 10^{-4}$ M)	Number of 30 min treatments	% of positive stained cells	Reference experiment
0	3	<1	Fig. 2 and Table 2
8.5	3	<5	Fig. 2
8.5	2	<5	Table 2

* The monolayers were checked after TEER experiments shown in Fig. 2 and Table 2.

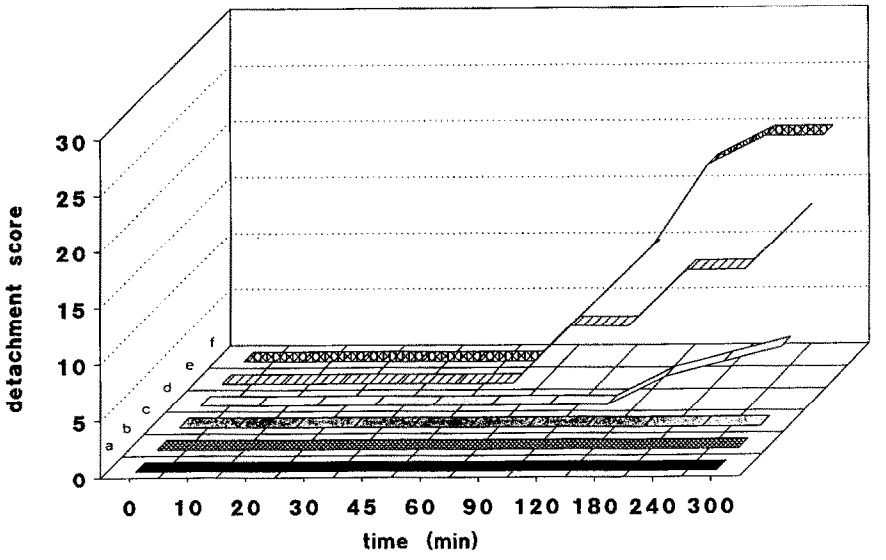

Fig. 6. Effect of continuous exposure to various concentrations of verapamil on cell detachment. a, Control; b, 1×10^{-4} M; c, 3×10^{-4} M; d, 7×10^{-4} M; e, 8.5×10^{-4} M; f, 10×10^{-4} M verapamil, respectively; all N = 4.

present in a concentration of 7×10^{-4} M, Flu could be seen in the paracellular route (Fig. 8), while no fluorescence inside the cells could be observed. The same holds true when FD-4 is used together with 7×10^{-4} M verapamil (Fig. 9). Clearly, verapamil enhances transport via the paracellular route.

DISCUSSION

The results of our studies show that racemic verapamil influences the paracellular permeability of the hydrophilic model compounds fluorescein and FD-4. The effect is reversible (Figs 1 and 2) and decreases with time following washing, as indicated by the non-linear relationship between the cumulative-cleared volume of the acceptor chamber and time (Figs 4 and 5). The effect observed following a second administration of verapamil indicates that the cells become increasingly sensitive to verapamil following repeated treatment, a maximum being

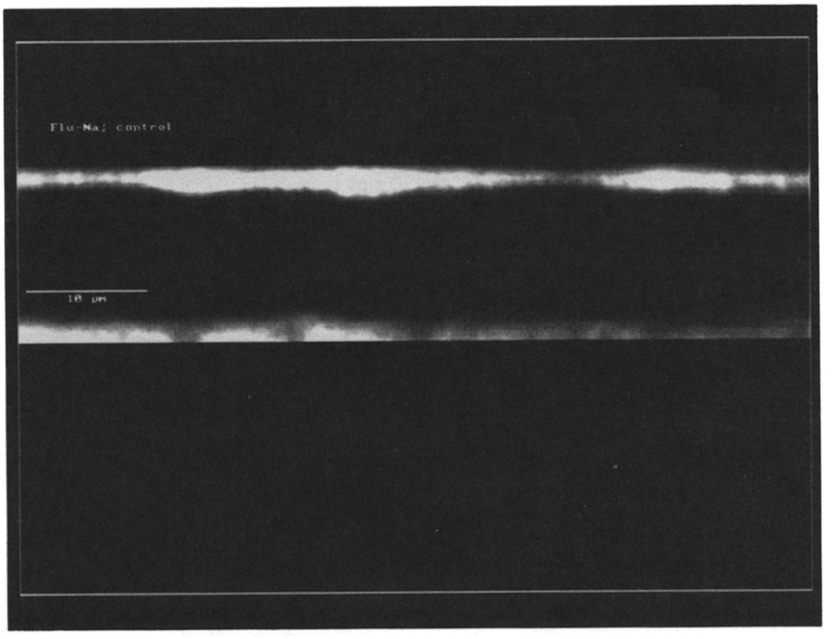

Fig. 7. *XZ* cross-section (i.e. perpendicular to the filter membrane) through Caco-2 monolayer incubated apically with Flu, without verapamil. Note the absence of paracellular fluorescence (top = apical, bottom = basolateral side).

reached at a time interval of 6 hr between first and second administration (Table 2). The experiments show that the enhancing effect of verapamil occurred only in a narrow concentration range ($>7 \times 10^{-4}$ M), where no direct association with cytotoxicity is involved for the following reasons: (i) the effect of 30 min exposure to verapamil on TEER was completely reversed after washing (Figs 1 and 2); (ii) the cells remained viable (trypan blue exclusion and propidium iodide exclusion); and (iii) no cell detachment occurred during the 30 min treatments with 7×10^{-4} M verapamil (Fig. 6).

All experiments indicate that verapamil enhanced hydrophilic drug transport across Caco-2 cell monolayers mainly via the paracellular route. Only when a drop in TEER was observed (Fig. 1) (reported to reveal changes mainly in the aqueous paracellular route [29, 30]) was the transport of the hydrophilic compounds Flu and FD-4 increased remarkably, as shown in Fig. 4 and Table 3. It would be interesting to see whether the transport of compounds larger than the ones used in these studies also increases after verapamil treatment and thereby to investigate the degree of opening of tight junctions by verapamil, as was done previously with the chelating compound EDTA [12].

The reversibility in effect, as seen in the reversibility of the TEER after removal of verapamil, was also observed in the transport experiments where the increased transport profiles showed a non-linear increasing curve. This indicates a decreasing clearance with time and thus a decreasing rate of transport of the hydrophilic compounds with time, and is consistent with the hypothesis that the opened tight junctions were closing after the removal of verapamil. This hypothesis was verified by means of confocal laser scanning microscopy. This technique allows vertical optical sectioning through living Caco-2 cell monolayers without fixation and therefore visualization of the transport route of the fluorescent compound. The fluorescence markers Flu and FD-4 were visualized only in the location of the paracellular route in Caco-2 cell monolayers treated with verapamil, at a concentration that enhanced transport of Flu and FD-4 (Figs 8 and 9, respectively); however, even the smallest compound (Flu) is restricted to the apical side of the monolayer when incubated without verapamil (Fig. 7).

There is no full explanation for the increment of TEER when verapamil was applied at a concentration of 1×10^{-4} M to 3×10^{-4} M (Table 1). This result is analogous to the results found by González-Marsical *et al.* [30], who reported that in Madin-Darby canine kidney (MDCK) cells, 1×10^{-5} M verapamil caused an increment in TEER. These authors did not provide a clear statement as to the underlying mechanism involved.

The concentration of verapamil used in these studies went from 1×10^{-4} M to 1×10^{-3} M. A concentration greater than 7×10^{-4} M verapamil not only caused a profound drop in TEER but also enhanced the transport of hydrophilic model compounds. These concentrations seem to be relatively high. However, in non-contractile tissue (e.g. colon, ileum, jejunum and duodenum in rats), the inhibition of calcium uptake has been shown to take place at concentrations from 1×10^{-4} M to 1.5×10^{-3} M [17, 18, 20] and thus justifies the

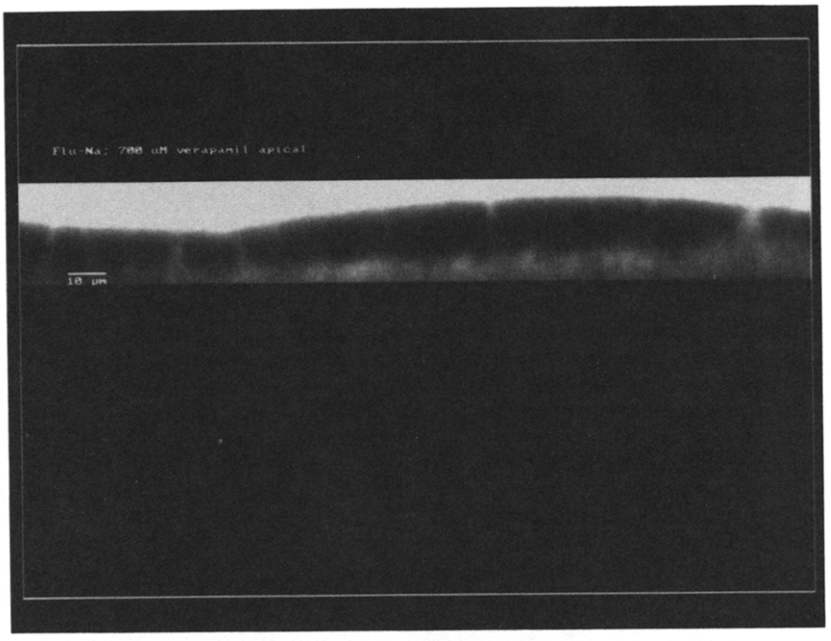

Fig. 8. *XZ* cross-section (i.e. perpendicular to the filter membrane) through Caco-2 monolayer incubated apically with Flu and 7×10^{-4} M verapamil. Note paracellular fluorescence (top = apical, bottom = basolateral side).

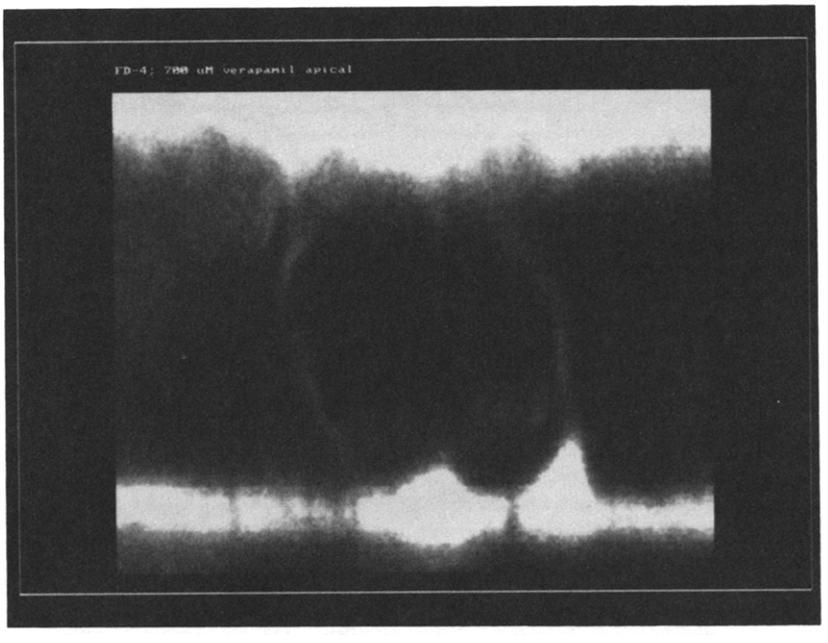

Fig. 9. *XZ* cross-section (i.e. perpendicular to the filter membrane) through Caco-2 monolayer incubated apically with FD-4 and 7×10^{-4} M verapamil. Note paracellular fluorescence (top = apical, bottom = basolateral side).

magnitude of the verapamil concentrations in the present series of experiments using intestinal Caco-2 cell monolayers.

In the data presented, we used racemic (±) as well as the *R*- and *S*-enantiomers of verapamil. Only the *S*-stereoisomer has been reported to induce calcium blocking but none of the sodium channel-blocking properties expressed by racemic verapamil [31]. Donowitz *et al.* [19, 20] demonstrated that the *S*-stereoisomer has slightly stronger quantitative effects on ileal electrolyte transport compared to the racemic mixture. However, a significant stronger

effect of the *S*-stereoisomer was not observed here (Fig. 3). Although the precise biochemical mechanism of the transport enhancement properties of verapamil is indeed within the limits of question, it may be speculated from these results that the induction of a water flow and thus absorption via the "solvent-drag" mechanism is responsible for the observed effects [19–21], although we did not measure an increased water flow. The relationship between the effect of verapamil, especially the mobilization of intracellular calcium and other factors correlated to tight junctional functions, should be studied in greater detail.

In summary, extrapolating from our observations it may be hypothesized that verapamil might be applied as an absorption enhancer for poorly absorbed hydrophilic drugs. Further studies will be necessary to investigate the underlying process of absorption enhancement by this compound as well as the applicability and safety of this drug as an absorption-enhancing compound.

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