

Sodium Cromoglycate Inhibits Absorption of the Major Soybean Allergen, Gly m Bd 30K, in Mice and Human Intestinal Caco-2 Cells¹

Thanakorn Weangsripanaval,² Kaeko Murota,³ Yoko Murakami,² Masaru Kominami,² Tatsuya Kusudo,² Tatsuya Moriyama,⁴ Tadashi Ogawa,⁵ and Teruo Kawada^{2*}

²Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan; ³Department of Food Science, Graduate School of Nutrition and Bioscience, The University of Tokushima, Tokushima 770-8503, Japan; ⁴Department of Applied Cell Biology, Graduate School of Agriculture, Kinki University, Nara 631-8505, Japan; and ⁵Department of Nutritional Science for Well-Being, Faculty of Health Science for Welfare, Kansai University of Welfare Science, Kashiwara, Osaka 582-0026, Japan

Abstract

Our previous data showed that Gly m Bd 30K was absorbed from the gastrointestinal tract and circulated in blood in mice. This study was conducted to determine the mechanism and identify the inhibitor of such absorption. Using sandwich ELISA and immunoblotting, we found that intact Gly m Bd 30K was absorbed from apical to basolateral solutions and intracellularly accumulated by Caco-2 cells in a dose- and time-dependent manner. The absorption and intracellular accumulation of Gly m Bd 30K were significantly suppressed when Caco-2 cells were treated with sodium cromoglycate (SCG) (0–50 mmol/L) in a dose-dependent manner. In 24-d-old mice orally treated with SCG (10–1000 mg/kg body weight), plasma Gly m Bd 30K concentration decreased significantly 30–120 min after Gly m Bd 30K (2000 mg/kg body weight) administration. Moreover, inhibitors that suppress the clathrin-dependent endocytosis dansylcadaverine, the caveolae-dependent endocytosis nystatin and clathrin, and the caveolae-dependent endocytosis methyl- β -cyclodextrin had inhibitory effects on the absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. These data indicate that Gly m Bd 30K is absorbed and intracellularly accumulated in Caco-2 cells via clathrin- or caveolae-dependent endocytosis. We propose that the absorption and intracellular accumulation of Gly m Bd 30K are inhibited by SCG via clathrin- or caveolae-dependent endocytosis. J. Nutr. 136: 2874–2880, 2006.

Introduction

Food allergies represent an important health problem that is continuously increasing in developed countries. It is estimated that food allergies affect ~8% of children and ~2% of the adult population (1). Relatively few food products are to blame for a large majority of allergic reactions, with most reactions attributed to peanuts, milk, eggs, wheat, crustaceans, soybeans, buckwheat, and rice. Since the FDA began allowing food products containing soybean proteins to carry a label promoting their health benefits, the consumption of many varieties of soybean products, including soybean beverages, tofu, soybean-based meat alternatives, and soybean protein powder, has increased in the United States and other developed countries. The increasing use of soybean products in processed foods poses a potential threat to soybean-sensitive individuals. Ogawa et al. (2) found that ~15 soybean proteins bind to IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis. We reported

that Gly m Bd 30K, which is recognized by IgE antibodies in >65% of soybean-sensitive patients, is the major allergenic protein in soybeans. Gly m Bd 30K is a soybean seed vacuolar 34-kDa oil-body-associated glycoprotein, P34, which is a member of the thiol protease family of the papain superfamily, and is highly homologous to the house dust mite allergen Der p 1 (3,4).

The intestinal epithelium is the primary site for dietary component absorption, including food allergens. We found that intact and digested fragments of Gly m Bd 30K are absorbed from the gastrointestinal tract and circulated in blood in mice after oral administration with purified and natural forms of Gly m Bd 30K in soy milk (5). The absorption of Gly m Bd 30K is age-dependent and enhanced in postweaned mice. When Gly m Bd 30K is coadministered with corn oil and sucrose fatty acid ester as an exogenous emulsifier, absorption is enhanced. However, the relevance of these findings in animal models to humans is not clear, because little is known about how and to what extent Gly m Bd 30K enters the systemic circulation after ingestion. In addition, studies on humans are rare, because food allergy is a life-threatening hypersensitivity reaction. For these reasons, the use of a human cell culture as an in vitro model of intestinal absorption would be a useful complementary approach to characterizing the mechanism of and the factors

¹ Supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 12460059) and the Brand Nippon Project (high-quality soybean team).

* To whom correspondence should be addressed. E-mail: fat@kais.kyoto-u.ac.jp.

that regulate the intestinal absorption of the soybean allergen Gly m Bd 30K. The human colon adenocarcinoma cell line (Caco-2 cells) is a useful model for studying the metabolism and transport of drugs and dietary compounds by intestinal absorptive cells (6,7).

Sodium cromoglycate [SCG;⁶ Intal or 5,5'-[2-hydroxy-1,3-propanediyl]bis(oxy)bis[4-oxo-4H-1-benzopyran-2-carboxylic acid disodium salt], developed from bis-chromone carboxylic acid, is an effective and safe drug for the prevention of bronchial asthma and food allergy (Fig. 1) (8–10). Chromone has been isolated from the seeds of the plant *Ammi visnaga* that grows wild in many Eastern Mediterranean countries. The chromone structure is similar to the chemical structure of flavonoids, a group of natural substances that are of current interest because of their antioxidant activity. Several studies have found that SCG functions in mast cell stabilization by inhibiting the release of chemical mediators, including histamine and the TNF, from mast cells (11–14). Additionally, SCG blocks the activation and subsequent mediator release from various inflammatory cells in addition to mast cells, including eosinophils, neutrophils, monocytes, alveolar macrophages, and lymphocytes. Studies on the function of SCG using human cells and tissues have shown that SCG suppresses the recruitment, activation, or responsiveness of inflammatory cells involved in the asthmatic cascade, possibly by altering the expression of various cytokines and leukocyte-specific adhesion molecules (15–19). However, the molecular mechanisms of how SCG prevents food allergy, underlying the gastrointestinal absorption of a food allergen, remain unclear.

In this study, we investigated the absorption and intracellular accumulation of Gly m Bd 30K in Caco-2 cells and measured the amounts of Gly m Bd 30K by sandwich ELISA and immunoblotting, using specific antibodies. We found that SCG inhibits allergen absorption by Caco-2 cells and young mice.

Materials and Methods

Gly m Bd 30K purification. The major soybean allergen Gly m Bd 30K was purified from the oil body pad of soybeans as described previously (5). Briefly, the oil body pad was isolated from soybean seeds that were soaked in water for 24 h, by homogenization in a solution (pH 8.6) of 0.1 mol/L Tris-HCl and 2 mmol/L mercaptoethanol, using a Polytron homogenizer. The homogenate was filtered through 3 layers of gauze and then centrifuged at $15,000 \times g$ for 20 min. The oil body pad was resuspended in the homogenization buffer and recentrifuged. Afterwards, it was resuspended in a solution (pH 8.6) containing 0.1 mmol/L NaCl, 0.1 mol/L Tris-HCl and 2 mmol/L mercaptoethanol and recentrifuged as described above. The oil body pad was then suspended and centrifuged in the same buffer again. Next, the oil body pad was resuspended in 1 mol/L Na_2CO_3 and allowed to stand for 3 h at 4°C. The supernatant was recovered by centrifugation as described above. The total protein in the supernatant was precipitated with 70% saturated ammonium sulfate. The precipitate was collected, dissolved in PBS, and dialyzed against the same buffer for 20 h at 4°C. For the absorption experiment using Caco-2 cells, Gly m Bd 30K in PBS was subjected to ion-exchange chromatography with HBSS, pH 7.4 (GIBCO), as eluent using a PD-10 column (Amersham Bioscience). The extract was stored at -28°C until use.

Cell culture. Caco-2 cells were obtained from the American Type Culture Collection and grown as described previously (20,21). Briefly, the culture medium contained Dulbecco's modified Eagle's medium (Sigma) supplemented with 100 mL/L fetal bovine serum (JRH Biosciences) and 10 mL/L nonessential amino acid solution (ICN Biomedicals).

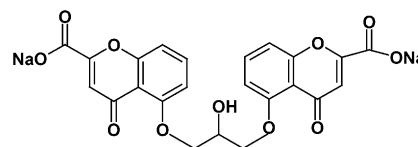


Figure 1 Chemical structure of SCG.

Monolayer cultures of the cells were grown in a humidified atmosphere of 5% CO_2 at 37°C . The cells were subcultured up to 70–80% confluence. For all studies, Caco-2 cells were seeded in Transwell inserts (polycarbonate membrane, 12-mm diameter and $0.4\text{-}\mu\text{m}$ pore size, Corning Costar) in 12-well plates at a density of 3.4×10^5 cells/insert ($1.12\text{ cm}^2/\text{insert}$). The basolateral (serosal) and apical (mucosal) compartments contained 1.5 and 0.5 mL of culture medium, respectively. The culture medium was replaced 3 times/wk.

Measurement of transepithelial electrical resistance of Caco-2 monolayers. The transepithelial electrical resistance (TEER) of Caco-2 monolayers was measured according to the method of Hidalgo et al. (22) with a Millicell-ERS instrument (Millipore). The measurement was performed before and after 2, 4, 6, and 8 h of incubation with Gly m Bd 30K.

Absorption and intracellular accumulation by Caco-2 cells. Caco-2 cells at passages 40–60 were used for all the experiments. To evaluate paracellular permeability with varying culture period, ^{14}C -mannitol was used as paracellular marker. Caco-2 cells were grown on Transwell inserts for 2, 7, 14, and 21 d. The inserts were washed 3 times with 37°C HBSS and incubated for 20 min in a CO_2 incubator before the addition of ^{14}C -mannitol ($9.1\text{ }\mu\text{mol/L}$, 0.5 mCi/L) to the apical solutions of the inserts (0.5 mL). Basolateral solutions were collected at 4 h. Radioactivity was measured using a Beckman Coulter LS 6500 multi purpose liquid scintillation counter (Beckman).

The absorption of Gly m Bd 30K was monitored for 14 d. For the dose-dependence experiment, Gly m Bd 30K ($62.5\text{--}1000\text{ }\mu\text{g}$) in HBSS was added to the apical solutions of the inserts (0.5 mL); the basolateral solutions contained HBSS (1.5 mL) and were incubated for 8 h in CO_2 incubator at 37°C . For the time-course experiment, the apical solutions were incubated with Gly m Bd 30K ($1000\text{ }\mu\text{g}$) for 2–24 h at 37°C . After the apical and basolateral solutions were collected at the designated times, the intracellular extract was prepared by washing all the inserts 3 times with ice-cold PBS. An acid-wash technique (23) was then used to remove cell surface-bound Gly m Bd 30K. Caco-2 cells were incubated for 10 min with 0.5 mL of ice-cold acetate-barbital buffer (28 mmol/L , 120 mmol/L NaCl and 20 mmol/L barbital; pH 3.0) on the apical solutions of inserts. The buffer was then removed and Caco-2 cells were washed 5 times with 1 mL of the same buffer. Then the cells were again washed 3 times with ice-cold PBS and trypsinized with 0.3 mL of 2.5 g/L trypsin and 1 mmol/L EDTA solutions (Nacalai Tesque). Cells in trypsin solution was added to a tube containing a culture medium for inactivating trypsin. The cell pellet was recovered by centrifugation at $100 \times g$ for 5 min and resuspended in 0.2 mL of HBSS. The intracellular extract was sonicated using a Microson ultrasonic cell disruptor (MISONIX) and stored at -20°C until analysis.

To determine the effects of SCG on the absorption and intracellular accumulation of Gly m Bd 30K and ^{14}C -mannitol, HBSS containing SCG ($0\text{--}50\text{ mmol/L}$) was added to the apical solutions of the inserts. After incubation for 30 min, the apical solutions were removed and then 0.5 mL of the mixture of SCG ($0\text{--}50\text{ mmol/L}$) and Gly m Bd 30K ($1000\text{ }\mu\text{g}$) or ^{14}C -mannitol ($9.1\text{ }\mu\text{mol/L}$, 0.5 mCi/L) was added to the apical solutions of the inserts. The inserts were incubated with Gly m Bd 30K for 8 h and with ^{14}C -mannitol for 4 h.

For the various endocytosis inhibitors, the inserts were washed 3 times with 37°C HBSS and incubated for 20 min at 37°C in a CO_2 incubator. Dansylcadaverine (Sigma) at 0.5 mmol/L , nystatin (Sigma) at 5 mg/L , or M β CD (Sigma) at 10 mmol/L in HBSS was added to the apical solutions of the inserts (0.5 mL); the basolateral solutions contained HBSS (1.5 mL). Transwells were incubated for 30 min at 37°C in a CO_2 incubator. Next, a mixture of an inhibitor at the concentration

⁶ Abbreviations used: BW, body weight; IAP, intestinal alkaline phosphatase; M β CD, methyl- β -cyclodextrin; SCG, sodium cromoglycate; TEER, transepithelial electrical resistance.

indicated above and Gly m Bd 30K (1000 μ g) in HBSS was added to the apical solutions of the inserts (0.5 mL). The inserts were incubated for 8 h at 37°C in a CO₂ incubator.

Effect of SCG on gastrointestinal absorption of Gly m Bd 30K in mice. The gastrointestinal absorption of Gly m Bd 30K in mice was examined as described previously (5). Briefly, male ddY mice (24-d-old, weighing 10–12 g) were purchased from Japan SLC. They were housed in a room with a 12:12 h light:dark cycle. The temperature and humidity were maintained at 23°C and within 48–62%, respectively. All the mice were fed a commercial diet (CRF-1; Oriental Yeast) (5). The experimental design was in accordance with the guidelines for animal experimentation and was approved by the Animal Experimental Committee of Kyoto University. The mice were food-deprived but had free access to water 24 h prior to the experiment. Using a 20-gauge needle equipped with a curved bulb-ended gavage tube, mice were orally administered 1 mL of PBS containing Gly m Bd 30K (2000 mg/kg BW) as the control condition ($n = 5$ /group). In the SCG treatment, the food-deprived mice ($n = 5$ /group) were orally administered 0.5 mL of dH₂O containing SCG (10, 100, or 1000 mg/kg BW). After 30 min, they were orally administered 1 mL of PBS containing Gly m Bd 30K (2000 mg/kg BW). Using a capillary tube, blood (0.15 mL) was drawn from the ophthalmic venous plexus of the anesthetized mice at 0, 30, 60, 90, and 120 min and placed in heparinized containers. Blood was immediately centrifuged at 3000 $\times g$ for 5 min at 4°C and plasma was stored at –20°C until analysis. Plasma Gly m Bd 30K concentration was determined by sandwich ELISA as described previously (5).

Cell proliferation assay. Caco-2 cells were seeded at a density of 3 $\times 10^5$ cells/cm² in each well of a 24-well plate and incubated for 14 d. One milliliter of medium containing SCG (0–50 mmol/L) and various endocytosis inhibitors was added in each well. Triton X-100 [0.1% (vol:vol)] was used as positive control. The plate was incubated for 72 h in a CO₂ incubator. The assay was performed using a CellTiter 96 Aqueous One Solution cell proliferation assay (Promega), according to the manufacturer's protocol. Briefly, the plate was incubated for 30 min with 400 μ L of a 1:9 diluted Cell Titer Aqueous One Solution reagent in culture's medium. The absorbance at 490 nm of each well was measured using a microplate reader (BioRad). The absorbance of the untreated cells was set as 100% and the viability of SCG and inhibitor treated cells was expressed as the percentage of untreated cells.

Electrophoresis and immunoblotting. Each basolateral solution or intracellular extract was mixed with SDS sample buffer. The reaction mixture was boiled for 5 min and analyzed by SDS-PAGE on 17.5% polyacrylamide gel, according to the method of Laemmli (24). For immunoblotting, the fractionated proteins from SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes under semidry conditions (25), and the electroblotted membranes were blocked in 5% skim milk in PBS, pH 7.4, containing 0.1% (vol:vol) Tween 20 (PBST). The membranes were incubated with a mouse monoclonal antibody against Gly m Bd 30K diluted 1:5000 in blocking solution for 1 h at room temperature. The bound IgG antibody was detected using the HRP-conjugated anti-mouse IgG antibody and an enhanced chemiluminescence Western blotting reagent (Amersham Bioscience).

ELISA. Gly m Bd 30K in each basolateral solution and intracellular extract was measured by ELISA. ELISA plates (96 wells, Asahi Technoglass) were incubated with 10 mg/L mouse monoclonal antibody against Gly m Bd 30K overnight at 4°C and blocked with Zepto-Block (ZeptoMetrix) for 2 h at room temperature. The wells were washed with PBST. Gly m Bd 30K, used as standard, was mixed with HBSS at 0–20 μ g/L. Next, 100 μ L each standard of Gly m Bd 30K in HBSS, basolateral solutions, and intracellular extracts were applied to each well of monoclonal antibody-coated plates, and the plates were incubated for 1 h at room temperature. The wells were washed with PBST, and 100 μ L of a 1:10,000-diluted rabbit polyclonal antibody against Gly m Bd 30K in PBS was added; the plates were then incubated for 1 h at room temperature. After the plates were washed with PBST, they were incubated with 100 μ L of a 1:10,000-diluted biotinylated anti-rabbit IgG in PBST. The plates were incubated with 100 μ L of a 1:10,000-diluted HRP-

conjugated AMDEX streptavidin (Amersham Bioscience) in PBS. Each well was washed 7 times with PBST and incubated with 100 μ L of TMB Microwell peroxidase substrate (Kirkegaard and Perry Laboratories) for 1 min; 100 μ L of 1 mol/L phosphoric acid was then added in the well. The absorbance at 450 nm was measured using a microplate reader (BioRad).

Quantification of intestinal alkaline phosphatase mRNA expression. By using M-MLV Reverse Transcriptase (Invitrogen), total RNA was reverse-transcribed according to the manufacturer's instructions with a Takara PCR Thermal Cycler SP. To quantify mRNA expression, PCR was performed using a fluorescence temperature cycler (LightCycler system). The oligonucleotide primer sets of intestinal alkaline phosphatase (IAP)-target genes were designed using a PCR primer selection program on the website of the Virtual Genomic Center from the GenBank database as follows: human IAP (NM001631), forward, GCAACCCTGCAACCCACCCAAGGAG; reverse, CCAGCATCCA-GATGTCCCGGGAG. To compare mRNA expression level among the samples, the copy numbers of all transcripts were divided by that of human 36B4 showing a constant expression level. Amplification was performed according to a published protocol (26). Briefly, 18 μ L of the reaction mixture contained 2.4 μ mol/L MgCl₂, 1.4 μ L of LightCycler DNA Master SYBR Green I dye, and 1 μ mol/L of each primer. The standard amplification program included 30 cycles of 3 steps each, which involved heating the product to 95°C at 20°C/s with a 30-s hold, annealing at 55°C at 20°C/s with a 5-s hold, and a final extension at 72°C at 20°C/s with a 10-s hold. The fluorescence at 530 nm was recorded on line at the end of the extension phase. Standards of PCR products amplified from the mRNA of Caco-2 cells were prepared by the same method as described above, using the LightCycler system. Each PCR product was subcloned into T-easy vectors (Promega) and used as standard plasmids for standard templates. The copy number of each standard plasmid was calculated from the absorbance at 260 nm and the molecular weight of each plasmid. The copy numbers of standards and samples were amplified simultaneously using the LightCycler system. The first cycle number indicated the specific fluorescence against noise, and the logarithm of the concentration of the PCR product standard from the external standard curve were calculated with the LightCycler software, version 3. To confirm the amplification of specific transcripts, melting curve profiles were produced at the end of each run.

Quantification of IAP activity. Caco-2 cells cultured in Transwells were washed 3 times with PBS and trypsinized with 0.3 mL of 2.5 g/L trypsin and 1 mmol/L EDTA solution. The cell suspension was added to the tube containing the culture medium to inactivate trypsin. The cell pellet was recovered by centrifugation at 100 $\times g$ for 5 min and resuspended in 0.2 mL of PBS containing a protease inhibitor cocktail (Nacalai Tesque). The method used to measure IAP activity was modified from Schlesinger et al. (27). Fifty microliters of intracellular extract was added to 0.75 mL of 100 mmol/L Tris-HCl (pH 8.0) containing 0.2 g/L *p*-nitrophenyl phosphate. After incubation for 5 min, 0.2 mL of 10% K₂HPO₄ was added to stop the reaction, and optical density, determined as the initial rates of *p*-nitrophenol production, was measured at 410 nm. One unit of enzyme activity was the amount of protein that produced a change in absorbance of 1.0/min, measured in a cuvette of 1-cm path length.

Protein assay. Protein concentration was measured by the Bradford method (28) with a protein assay kit (BioRad) using bovine γ -globulin (Sigma) as a standard.

Statistical analysis. Data are presented as mean $\pm$ SD. The differences in the amounts of Gly m Bd 30K in basolateral solutions and intracellular extracts between the control and SCG treatments or among various endocytosis inhibitors were analyzed by 1-way ANOVA and a post-hoc Dunnett's test. The differences in the plasma Gly m Bd 30K concentration among the groups for each time point were analyzed by 1-way ANOVA with a Tukey-Kramer Multiple Comparison test. The difference in plasma Gly m Bd 30K concentration from that of the previous time point in that group were analyzed by a paired *t*-test (5). The StatView program (SAS Institute) was used for the analysis. Differences were considered significant at $P < 0.05$.

Results

Changes in TEER, activity and mRNA expression of IAP, and permeability of ^{14}C -mannitol during Caco-2 cell proliferation and differentiation. Caco-2 cells grown on permeable membrane filters reached confluence after 3–4 d. The TEER increased, reached a plateau after 8–10 d, and remained constant up to 21 d. The Caco-2 cells used in this study gave TEER between 600 and 900 Ω/cm^2 . IAP is exclusively expressed in enterocytes of the small intestine and hence serves as an excellent specific marker of crpt-villus differentiation (29). In this study, we examined the activity and mRNA expression of IAP in Caco-2 cells cultivated for 2, 7, 14, and 21 d on permeable membrane filters. The IAP activities were 0.119 ± 0.038 , 0.330 ± 0.025 , 0.411 ± 0.028 , and 0.406 ± 0.007 unit/mg protein on days 2, 7, 14, and 21, respectively. Using LightCycler real-time PCR, the mRNA expression level of IAP also increased to 913 ± 182 , 843 ± 122 , and $1000 \pm 61\%$ from d 2 to days 7, 14, and 21, respectively. The percentage permeabilities of paracellular marker ^{14}C -mannitol after 4 h of incubation with Caco-2 cells cultivated for days 7, 14, and 21 were 4.802 ± 1.013 , 1.705 ± 0.114 , and 1.945 ± 0.050 , respectively. These results indicate that Caco-2 cells in our system form a polarized monolayer that exhibit a well-defined tight junction between cells. We used Caco-2 cells cultivated for 14 d in subsequent experiments.

Dose and time dependences of absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. The absorption of Gly m Bd 30K from the apical to basolateral solutions was investigated in the dose range from 62.5 to 1000 $\mu\text{g}/\text{insert}$ at 8 h after incubation. Using sandwich ELISA, Gly m Bd 30K was detected in the basolateral solutions in a dose-dependent manner [5.55 ± 1.02 $\mu\text{g}/\text{insert}$ after incubation with Gly m Bd 30K (1000 $\mu\text{g}/\text{insert}$)] (Fig. 2A). Intact Gly m Bd 30K was detected in each basolateral solution and intracellular extract by immunoblotting (Fig. 2B,D). The intracellular accu-

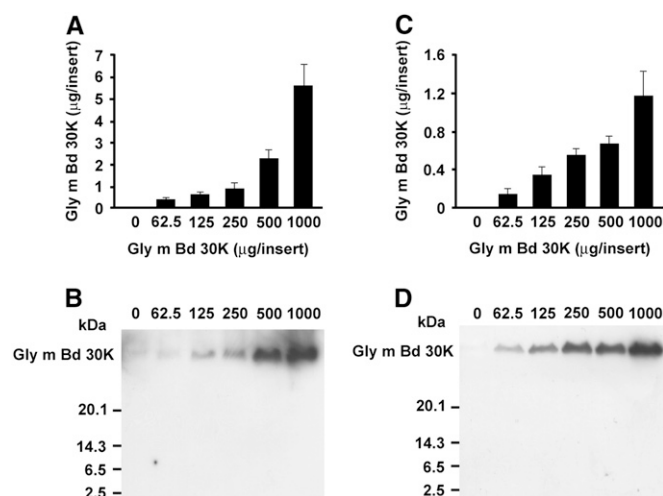


Figure 2 Dose-dependences of absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. Gly m Bd 30K at 62.5–1000 $\mu\text{g}/\text{insert}$ was incubated with Caco-2 cells for 8 h. The amounts of Gly m Bd 30K in the basolateral solutions (A) and intracellular extracts (C) were evaluated using sandwich ELISA. Immunoblots of the basolateral solutions (B) and intracellular extracts (D) were analyzed. Protein was loaded at 6 $\mu\text{g}/\text{lane}$. Data are means $\pm$ SD, $n = 4$ –6.

mulation of Gly m Bd 30K by Caco-2 cells also increased continuously (1.16 ± 0.25 $\mu\text{g}/\text{insert}$ after incubation at 1000 $\mu\text{g}/\text{insert}$) (Fig. 2C).

In the time course experiment, Gly m Bd 30K was incubated in the apical solutions at 1000 $\mu\text{g}/\text{insert}$ from 2 to 24 h at 37°C. The amounts of intact Gly m Bd 30K in the basolateral solutions continuously increased up to 8 h (5.55 ± 1.02 $\mu\text{g}/\text{insert}$) and remained constant thereafter up to 24 h (5.52 ± 0.61 $\mu\text{g}/\text{insert}$) (Fig. 3A,B). The intracellular accumulation of Gly m Bd 30K was enhanced in a time-dependent manner (1.72 ± 0.10 $\mu\text{g}/\text{insert}$ for 24-h of incubation) (Fig. 3C,D). These results indicate that intact Gly m Bd 30K is absorbed and intracellularly accumulated by Caco-2 cells in a dose- and time-dependent manner.

SCG inhibits Gly m Bd 30K absorption by Caco-2 cells and mice. After the treatment of Caco-2 cells with SCG (0–50 mmol/L), the absorption and intracellular accumulation of Gly m Bd 30K were inhibited by increasing SCG concentrations (Fig. 4). The inhibitory activity of SCG (50 mmol/L) on the absorption was $86.2 \pm 7.7\%$ ($P < 0.05$) and that on intracellular accumulation was $70.7 \pm 5.4\%$ ($P < 0.05$). The 50% inhibition (IC_{50}) values for absorption and intracellular accumulation occurred at SCG concentrations of 10.9 and 16.1 mmol/L, respectively. In the cell proliferation assay, SCG (50 mmol/L) did not affect the viability of Caco-2 cells.

To confirm that SCG inhibits Gly m Bd 30K absorption, we determined the effect of SCG in mice. In the 24-d-old control mice administered Gly m Bd 30K alone (2000 mg/kg BW), plasma Gly m Bd 30K was detected at its maximum concentration of 0.391 ± 0.055 mg/L 30 min after the administration ($P < 0.05$), and its concentration gradually decreased to 0.127 ± 0.052 mg/L by the end of the observation period (Fig. 5). In the SCG (10 mg/kg BW) treated mice, SCG inhibited the Gly m Bd 30K absorption at 30 min, but not at 60–120 min, after the allergen challenge, compared with the control group ($P < 0.05$). In the mice treated with SCG at 100 or 1000 mg/kg BW, gastrointestinal Gly m Bd 30K absorption was concentration-dependently inhibited 30–120 min ($P < 0.05$) after the administration of Gly m Bd 30K (2000 mg/kg BW). Plasma Gly m Bd 30K concentration reached a plateau at 0.047 ± 0.01 mg/L and

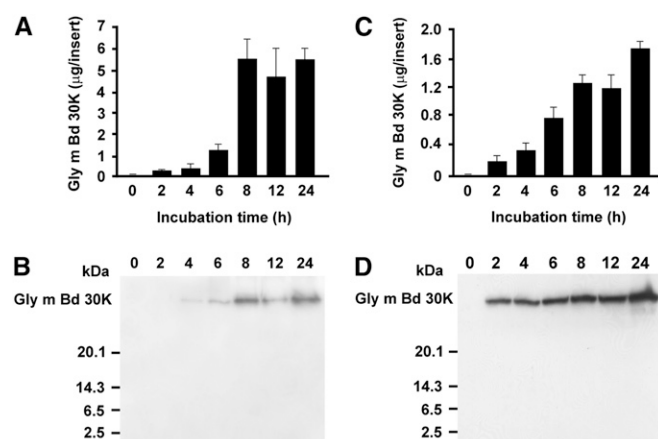


Figure 3 Time-courses of absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. Gly m Bd 30K at 1000 $\mu\text{g}/\text{insert}$ was incubated with Caco-2 cells for 2–24 h. The amounts of Gly m Bd 30K in the basolateral solutions (A) and intracellular extracts (C) were evaluated using sandwich ELISA. Immunoblots of the basolateral solutions (B) and intracellular extracts (D) were analyzed. Protein was loaded at 6 $\mu\text{g}/\text{lane}$. Data are means $\pm$ SD, $n = 4$.

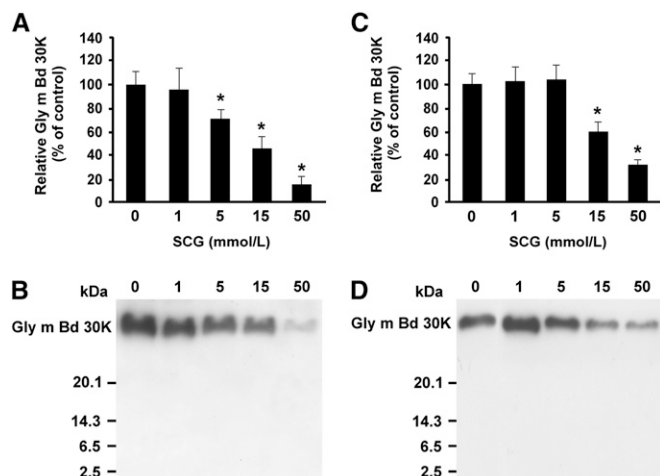


Figure 4 Effects of SCG on absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. Gly m Bd 30K at 1000 $\mu\text{g}/\text{insert}$ and SCG (0–50 mmol/L) were used. Caco-2 cells were preincubated with SCG for 30 min and remained in the apical solutions for the following 8 h of incubation with Gly m Bd 30K. The amounts of Gly m Bd 30K in the basolateral solutions (A) and intracellular extracts (C) were evaluated using sandwich ELISA. Immunoblots of the basolateral solutions (B) and intracellular extracts (D) were analyzed. Protein was loaded at 6 $\mu\text{g}/\text{lane}$. Data are means $\pm$ SD, $n = 4$ –6. *Different from the control group, $P < 0.05$.

0.017 ± 0.001 mg/L in mice administered doses of 100 and 1000 mg/kg BW, respectively, at 30 min.

SCG decreases ^{14}C -mannitol permeability. The permeability of ^{14}C -mannitol after a 4-h of incubation was decreased by SCG in a concentration-dependent manner (inhibited by $14.2 \pm 0.9\%$ for 15 mmol/L ($P < 0.05$) and $24.1 \pm 4.4\%$ for 50 mmol/L ($P < 0.05$)).

Effects of various endocytosis inhibitors on absorption and intracellular accumulation of Gly m Bd 30K. The following experiment was conducted to determine whether various endocytosis inhibitors affect the absorption and intracellular accumulation of Gly m Bd 30K. Dansylcadaverine (0.5 mmol/L) inhibited the absorption by $18.6 \pm 9.3\%$ ($P < 0.05$) and the intracellular accumulation by $45.9 \pm 17.9\%$ ($P < 0.05$) (Fig. 6). Nystatin (5 mg/L) did not affect the absorption but inhibited the

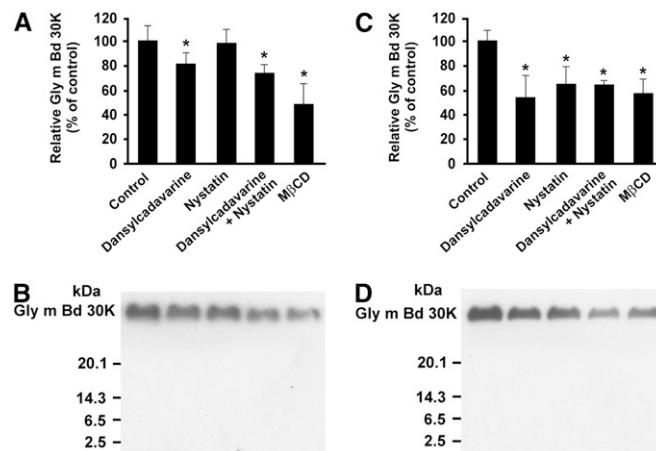


Figure 6 Effects of various endocytosis inhibitors on absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells. Gly m Bd 30K was at 1000 $\mu\text{g}/\text{insert}$, dansylcadaverine at 0.5 mmol/L, nystatin at 5 mg/L, and M β CD at 10 mmol/L. Caco-2 cells were preincubated with inhibitors for 30 min and remained in the apical solutions for the following 8 h of incubation in the presence of Gly m Bd 30K. The amounts of Gly m Bd 30K in the basolateral solutions (A) and intracellular extracts (C) were evaluated using sandwich ELISA. Immunoblots of the basolateral solutions (B) and intracellular extracts (D) were analyzed. Protein was loaded at 6 $\mu\text{g}/\text{lane}$. Data are means $\pm$ SD, $n = 4$ –12. *Different from the control group, $P < 0.05$.

intracellular accumulation by $35.6 \pm 13.4\%$ ($P < 0.05$). No further inhibition of the intracellular accumulation occurred when nystatin and dansylcadaverine were both included ($35.8 \pm 3.5\%$), compared with either dansylcadaverine or nystatin alone. M β CD (10 mmol/L) inhibited the absorption by $52.4 \pm 17.2\%$ ($P < 0.05$) and the intracellular accumulation by $42.8 \pm 11.1\%$ ($P < 0.05$). In the cell proliferation assay, inhibitors used in this study did not affect the viability of Caco-2 cells.

Discussion

Our previous in vivo study showed that both purified and natural forms of Gly m Bd 30K in soymilk were absorbed from the gastrointestinal tract and circulated in blood in young and older mice. We also demonstrated that the Gly m Bd 30K absorption was dose-dependent, decreased in extent with age, and was enhanced when coadministered with corn oil and a sucrose fatty acid ester. In this study, we demonstrated the molecular mechanisms underlying intestinal Gly m Bd 30K absorption and identified SCG as the allergen absorption inhibitor by Caco-2 cells as a model of absorptive enterocytes.

In Caco-2 cells, the absorption and intracellular accumulation of Gly m Bd 30K occur in a dose-dependent manner (Fig. 2). The percentage of absorption and intracellular accumulation are very low after 8 h of incubation with Gly m Bd 30K at 1000 $\mu\text{g}/\text{insert}$ (0.555 ± 0.102 and $0.116 \pm 0.025\%$, respectively). This suggests that Gly m Bd 30K is not absorbed through Caco-2 cells, by generating different Gly m Bd 30K concentrations among the apical and basolateral solutions or by simple passive diffusion. This study found that TEERs did not decrease for Gly m Bd 30K incubation until 24 h (data not shown). Reports indicate that TEER is highly affected by cytotoxicity, particularly cytotoxicity caused by membrane-perturbing toxicants, because a decrease in TEER is a clear indication of an increase in cell permeability caused by toxins enhancing tight-junction permeability (30–32). This finding suggests that Gly m Bd 30K

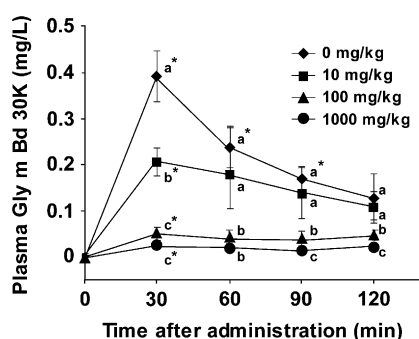


Figure 5 Effect of SCG on gastrointestinal absorption of Gly m Bd 30K in mice. Food-deprived 24-d-old mice were orally administered 0.5 mL of dH₂O containing SCG (10, 100, or 1000 mg/kg BW). After 30 min, they were orally administered 1 mL of PBS containing Gly m Bd 30K (2000 mg/kg BW). Data are means $\pm$ SD, $n = 5$. Means at a time without a common letter differ, $P < 0.05$. *Different from the previous time point in that group, $P < 0.05$.

dose not affect tight-junction integrity. From the above results, we conclude that intact Gly m Bd 30K is absorbed across Caco-2 cells via the transcellular pathway. This study found that Gly m Bd 30K in each basolateral solution and intracellular extract was the only intact protein (Figs. 2B,D and 3B,D). However, we did not detect fragments of molecular masses <2.5 kDa because we ran our SDS-PAGE experiments on a 17.5% polyacrylamide gel. This result was consistent with our previous finding in mice that Gly m Bd 30K is not digested by the protease expressed in the brush border membrane of an absorptive enterocyte or degraded during transport between absorptive cells. These unique properties could be important factors that make Gly m Bd 30K an allergenic protein for sensitizing allergic reactions.

SCG has been used for many years to prevent food allergic reactions. Several studies have shown that SCG affects the intestinal permeability of different-sized molecules, such as mannitol and lactulose (33–38). However, the molecular mechanisms of how SCG prevents food allergy using the soybean allergen are not clear. This study found that SCG inhibits the absorption and intracellular accumulation of Gly m Bd 30K in Caco-2 cells and mice (Figs. 4, 5). The IC_{50} values of SCG used in Caco-2 cells (10.9 mmol/L for the absorption and 16.1 mmol/L for the intracellular accumulation) are within the range of SCG concentrations (up to 1000 mg/kg, ~30 mmol/L) used to induce an inhibition effect on Gly m Bd 30K absorption in mice. In humans, <1% of the administered SCG dose is absorbed from the gastrointestinal tract. A total of 50% SCG is excreted unchanged in the bile and the other 50% in the urine. Fancoise-Andre et al. reported that the peak plasma SCG concentration occurred between 30 and 120 min and was within the range of 34–4.9 mg/L after the oral administration of 300 mg of SCG in healthy subjects (39–41). On the basis of this observation, we suggest that the effective SCG concentration in our study is close to the active concentration that can be found after the intake of the recommended dose (300 mg/d). Additionally, we found that the paracellular permeability of ^{14}C -mannitol is also inhibited by SCG in Caco-2 cells.

Studies using intestinal cell lines and animals showed that proteins such as IgG, HRP, and β -lactoglobulin are transported across intestinal cells via endocytosis. (42–44). In the time course experiment, the absorption of Gly m Bd 30K saturated after 8 h of incubation (Fig. 3A,B), but the intracellular accumulation was continuously enhanced up to 24 h of incubation (Fig. 3C,D). These results suggest that the intracellular carrier or transporter is associated with Gly m Bd 30K absorption. To clarify the molecular mechanisms on the absorption and intracellular accumulation of Gly m Bd 30K, various substances that interfere with endocytosis in Caco-2 cells were used. Dansylcadaverine, which blocks the formation of coated pits by inhibiting transglutaminase in the cell membrane was used as an inhibitor of receptor mediated–clathrin dependent endocytosis (23,45,46). Nystatin is a sterol-binding drug that inhibits caveolae-dependent endocytosis (47–50). $M\beta$ CD extracts cholesterol from the membrane and inhibits both clathrin- and caveolae-dependent endocytosis (51–55). We found that intracellular Gly m Bd 30K accumulation is reduced by ~50% by dansylcadaverine, nystatin, and $M\beta$ CD (Fig. 6). However, no marked inhibition occurred when dansylcadaverine and nystatin were both included, indicating that dansylcadaverine and nystatin have a nonsynergistic inhibitory effect via the same route. We found that $M\beta$ CD and dansylcadaverine are effective in inhibiting Gly m Bd 30K in the basolateral solutions and intracellular extracts. Our results suggest that almost all Gly m Bd 30K is absorbed and intracellularly accumulated through Caco-2 cells via receptor-

mediated clathrin- or caveolae-dependent endocytosis. Because the inhibitory effect of the intracellular accumulation was not >50%, we presume that a portion of Gly m Bd 30K is absorbed through other pathways via the intracellular route. However, this is still speculative; further studies are required to elucidate the molecular mechanism of Gly m Bd 30K absorption. We found that the nystatin affected intracellular Gly m Bd 30K accumulation, but not Gly m Bd 30K absorption, in the basolateral solutions, suggesting that protein release from intracellular to basolateral solutions is involved in Gly m Bd 30K transport.

In summary, we found that intact Gly m Bd 30K is dose- and time-dependently absorbed and intracellularly accumulated by Caco-2 cells via clathrin- or caveolae- dependent endocytosis. We demonstrated that SCG inhibits the absorption and intracellular accumulation of Gly m Bd 30K by Caco-2 cells and mice in a dose-dependent manner. These observations suggest that SCG is a potential allergen absorption inhibitor and can potentially prevent food allergic reactions.

Literature Cited

1. Jansen JJ, Kardinaal AF, Huijbers G, Vlieg-Boerstra BJ, Martens BP, Ockhuizen T. Prevalence of food allergy and intolerance in the adult Dutch population. *J Allergy Clin Immunol*. 1994;93:446–56.
2. Kalinski A, Weisemann JM, Matthews BF, Herman EM. Molecular cloning of a protein associated with soybean seed oil bodies that is similar to thiol proteases of the papain family. *J Biol Chem*. 1990;265:13843–8.
3. Ogawa T, Bando N, Tsuji H, Okajima H, Nishikawa K, Sasaoka K. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic dermatitis. *J Nutr Sci Vitaminol (Tokyo)*. 1991;37:555–65.
4. Ogawa T, Tsuji H, Bando N, Kitamura K, Zhu YL, Hirano H, Nishikawa K. Identification of the soybean allergenic protein, Gly m Bd 30K, with the soybean seed 34-kDa oil-body-associated protein. *Biosci Biotechnol Biochem*. 1993;57:1030–33.
5. Weangsriripana T, Moriyama T, Kageura T, Ogawa T, Kawada T. Dietary fat and an exogenous emulsifier increase the gastrointestinal absorption of a major soybean allergen, Gly m Bd 30K, in mice. *J Nutr*. 2005;135:1738–44.
6. Pinto M, Robine-Leon S, Appay MD, Keding M, Triadou N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffen K, et al. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol Cell*. 1983;47:323–30.
7. Grasset E, Pinto M, Dussaulx E, Zweibaum A, Desjeux JF. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am J Physiol*. 1984;247:C260–7.
8. Edwards AM, Howell JB. The chromones: history, chemistry and clinical development. A tribute to the work of Dr R. E. C. Altounyan. *Clin Exp Allergy*. 2000;30:756–74.
9. Storms W, Kaliner MA. Cromolyn sodium: fitting an old friend into current asthma treatment. *J Asthma*. 2005;42:79–89.
10. Van Elburg RM, Heymans HS, De Monchy JG. Effect of disodium cromoglycate on intestinal permeability changes and clinical response during cow's milk challenge. *Pediatr Allergy Immunol*. 1993;4:79–85.
11. Ginsburg MI, Baldwin AL. Disodium cromoglycate stabilizes mast cell degranulation while reducing the number of hemoglobin-induced microvascular leaks in rat mesentery. *Am J Physiol Heart Circ Physiol*. 2004;286:H1750–6.
12. Edwards AM. The discovery of cromolyn sodium and its effect on research and practice in allergy and immunology. *J Allergy Clin Immunol*. 2005;115:885–8.
13. Shichijo M, Inagaki N, Nakai N, Kimata M, Nakahata T, Serizawa I, Iikura Y, Saito H, Nagai H. The effects of anti-asthma drugs on mediator release from cultured human mast cells. *Clin Exp Allergy*. 1998;28:1228–36.
14. Patalano F, Ruggieri F. Sodium cromoglycate: a review. *Eur Respir J Suppl*. 1989;2:556–60.

15. Shin HY, Kim JS, An NH, Park RK, Kim HM. Effect of disodium cromoglycate on mast cell-mediated immediate-type allergic reactions. *Life Sci.* 2004;74:2877-87.
16. Bissonnette EY, Enciso JA, Befus AD. Inhibition of tumor necrosis factor- α (TNF- γ) release from mast cells by the anti-inflammatory drugs, sodium cromoglycate and nedocromil sodium. *Clin Exp Immunol.* 1995;102:78-84.
17. Cox JS. Disodium cromoglycate. Mode of action and its possible relevance to the clinical use of the drug. *Br J Dis Chest.* 1971;65:189-204.
18. Orr TS, Gwilliam J, Cox JS. Studies on passive cutaneous anaphylaxis in the rat with disodium cromoglycate. II. A comparison of the rat anti-dnp 7S 2 and rat reagin induced cutaneous reactions. *Immunology.* 1971;21:405-17.
19. Kimata H, Yoshida A, Ishioka C, Mikawa H. Disodium cromoglycate (DSCG) selectively inhibits IgE production and enhances IgG4 production by human B cell in vitro. *Clin Exp Immunol.* 1991;84:395-9.
20. Murota K, Storch J. Uptake of micellar long-chain fatty acid and sn-2-monoacylglycerol into human intestinal Caco-2 cells exhibits characteristics of protein-mediated transport. *J Nutr.* 2005;135:1626-30.
21. Murota K, Shimizu S, Miyamoto S, Izumi T, Obata A, Kikuchi M, Terao J. Unique uptake and transport of isoflavone aglycones by human intestinal caco-2 cells: comparison of isoflavonoids and flavonoids. *J Nutr.* 2002;132:1956-61.
22. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology.* 1989;96:736-49.
23. Sai Y, Kajita M, Tamai I, Wakama J, Wakamiya T, Tsuji A. Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells. *Am J Physiol.* 1998;275:G514-20.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680-5.
25. Kyhse-Andersen J. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods.* 1984;10:203-9.
26. Takahashi N, Kawada T, Goto T, Yamamoto T, Taimatsu A, Matsui N, Kimura K, Saito M, Hosokawa M, et al. Dual action of isoprenols from herbal medicines on both PPAR γ and PPAR α in 3T3-L1 adipocytes and HepG2 hepatocytes. *FEBS Lett.* 2002;514:315-22.
27. Schlesinger MJ. Formation of a defective alkaline phosphatase subunit by a mutant of *Escherichia coli*. *J Biol Chem.* 1967;242:1604-11.
28. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-54.
29. Hodin RA, Chamberlain SM, Meng S. Pattern of rat intestinal brush-border enzyme gene expression changes with epithelial growth state. *Am J Physiol.* 1995;269:C385-91.
30. Moore R, Pothoulakis C, Lamont JT, Carlson S, Madara JL. C. difficile toxin A increases intestinal permeability and induces Cl-secretion. *Am J Physiol.* 1990;259:G165-72.
31. Narai A, Arai S, Shimizu M. Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayer by cytotoxic membrane perturbants. *Toxicol In Vitro.* 1997;11:347-54.
32. Okada T, Narai A, Matsunaga S, Fusetani N, Shimizu M. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. *Toxicol In Vitro.* 2000;14:219-26.
33. Andre F, Andre C, Feknous M, Colin L, Cavagna S. Digestive permeability to different-sized molecules and to sodium cromoglycate in food allergy. *Allergy Proc.* 1991;12:293-8.
34. Andre C, Andre F, Colin L. Effect of allergen ingestion challenge with and without cromoglycate cover on intestinal permeability in atopic dermatitis, urticaria and other symptoms of food allergy. *Allergy.* 1989;44:47-51.
35. Andre C, Andre F, Colin L, Cavagna S. Measurement of intestinal permeability to mannitol and lactulose as a means of diagnosing food allergy and evaluating therapeutic effectiveness of disodium cromoglycate. *Ann Allergy.* 1987;59:127-30.
36. Falth-Magnusson K, Kjellman NI, Magnusson KE, Sundqvist T. Intestinal permeability in healthy and allergic children before and after sodium cromoglycate treatment assessed with different-sized polyethyleneglycols (PEG 400 and PEG 1000). *Clin Allergy.* 1984;14:277-86.
37. Falth-Magnusson K, Kjellman NI, Odelram H, Sundqvist T, Magnusson KE. Gastrointestinal permeability in children with cow's milk allergy: effect of milk challenge and sodium cromoglycate as assessed with polyethyleneglycols (PEG 400 and PEG 1000). *Clin Allergy.* 1986;16:543-51.
38. Cox JS. Disodium cromoglycate (FPL 670) ('Intal'): a specific inhibitor of reaginic antibody-antigen mechanisms. *Nature.* 1967;216:1328-9.
39. Walker SR, Evans ME, Richards AJ, Paterson JW. The fate of (14 C)disodium cromoglycate in man. *J Pharm Pharmacol.* 1972;24:525-31.
40. Moss GF, Jones KM, Ritchie JT, Cox JS. Plasma levels and urinary excretion of disodium cromoglycate after inhalation by human volunteers. *Toxicol Appl Pharmacol.* 1971;20:147-56.
41. Abrahamson DR, Rodewald R. Evidence for the sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. *J Cell Biol.* 1981;91:270-80.
42. Heyman M, Crain-Denoyelle AM, Nath SK, Desjeux JF. Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2. *J Cell Physiol.* 1990;143:391-5.
43. Marcon-Genty D, Tome D, Kheroua O, Dumontier AM, Heyman M, Desjeux JF. Transport of beta-lactoglobulin across rabbit ileum in vitro. *Am J Physiol.* 1989;256:G943-8.
44. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. *Mol Biol Cell.* 2004;15:176-88.
45. Schlegel R, Dickson RB, Willingham MC, Pastan IH. Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of alpha 2-macroglobulin. *Proc Natl Acad Sci USA.* 1982;79:2291-5.
46. Johannes L, Lamaze C. Clathrin-dependent or not: is it still the question? *Traffic.* 2002;3:443-51.
47. Decorti G, Malusa N, Furlan G, Candussio L, Klugmann FB. Endocytosis of gentamicin in a proximal tubular renal cell line. *Life Sci.* 1999;65:1115-24.
48. Chen Y, Norkin LC. Extracellular simian virus 40 transmits a signal that promotes virus enclosure within caveolae. *Exp Cell Res.* 1999;246:83-90.
49. Rothberg KG, Ying YS, Kamen BA, Anderson RG. Cholesterol controls the clustering of the glycosphospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J Cell Biol.* 1990;111:2931-38.
50. Schnitzer JE, Oh P, Pinney E, Allard J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J Cell Biol.* 1994;127:1217-32.
51. Torgersen ML, Skretting G, van Deurs B, Sandvig K. Internalization of cholera toxin by different endocytic mechanisms. *J Cell Sci.* 2001;114:3737-47.
52. Rodal SK, Skretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell.* 1999;10:961-74.
53. Ohtani Y, Irie T, Uekama K, Fukunaga K, Pitha J. Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes. *Eur J Biochem.* 1989;186:17-22.
54. Klein U, Gimpl G, Fahrenholz F. Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry.* 1995;34:13784-93.
55. Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci USA.* 1999;96:6775-80.