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Rabbit small intestine does not contain an annexin II/caveolin 1 complex as a target for 2-azetidinone cholesterol absorption inhibitors

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Abstract

Intestinal cholesterol absorption is specifically inhibited by the 2-azetidinone cholesterol absorption inhibitor ezetimibe. Photoreactive ezetimibe analogues specifically label a 145-kDa protein in the brush border membrane of enterocytes from rabbit small intestine identified as aminopeptidase N (CD13). In zebrafish and mouse small intestinal cytosol, a heterocomplex of M_r 52 kDa between annexin II and caveolin 1 was suggested as a target of ezetimibe. In contrast, in the cytosol and brush border membrane vesicles (BBMV) from rabbit small intestine of control animals or rabbits treated with the nonabsorbable cholesterol absorption inhibitor AVE 5530, both annexin II and caveolin 1 were exclusively present as monomers without any heterocomplex formation. Upon immunoprecipitation with annexin II a 52-kDa band was observed after immunostaining with annexin II antibodies, whereas no staining of a 52-kDa band occurred with anti-caveolin 1 antibodies. Vice versa, a 52-kDa band obtained by immunoprecipitation with caveolin 1 antibodies did not stain with annexin II-antibodies. The intensity of the 52-kDa band was dependent on the amount of antibody and was also observed with anti-actin or anti-APN antibodies suggesting that the 52-kDa band is a biochemical artefact. After incubation of cytosol or BBMV with radioactively labelled ezetimibe analogues, no significant amounts of the ezetimibe analogues could be detected in the immunoprecipitate with caveolin-1 or annexin II antibodies. Photoaffinity labelling of rabbit small intestinal BBMV with ezetimibe analogues did not result in labelling of proteins being immunoreactive with annexin II, caveolin 1 or a 52-kDa heterocomplex. These findings indicate that the rabbit small intestine does not contain an annexin II/caveolin 1 heterocomplex as a target for ezetimibe.

Keywords: Cholesterol absorption; Ezetimibe; Annexin II/caveolin1; Photoaffinity labelling; Brush border membrane (rabbit)

1. Introduction

Intestinal absorption of cholesterol contributes significantly to the serum cholesterol levels. The molecular mechanism of intestinal cholesterol absorption is still not understood; the experimental findings with specific cholesterol absorption inhibitors [1,2], sterol specificity [3] and profound species differences [4] strongly favour a protein-mediated process. A number of putative candidates for an intestinal cholesterol transporter have been described [5–9], but for none of these proteins has clear evidence as an intestinal cholesterol uptake system been presented. Ezetimibe is a cholesterol absorption inhibitor which is rapidly absorbed in the upper small intestine; a glucuronide formed in the intestine is secreted with bile into the lumen of the small intestine where it exerts its pharmacological activity and inhibits intestinal cholesterol absorption [10]. The existence of highly specific cholesterol absorption inhibitors with clear structure–activity relationships prompted us to identify the proteins involved in cholesterol absorption with photoreactive analogues of the cholesterol absorption inhibitor ezetimibe [11–13] and of cholesterol [14]. A 145-kDa integral membrane protein was identified as the specific binding protein for cholesterol absorption inhibitors in the brush border membrane of small intestinal enterocytes [11–13,15–17], whereas cholesterol specifically interacts with a 80-kDa membrane protein [15,16] suggesting that cholesterol uptake occurs by a complex protein machinery rather than by a single transporter. The 145-kDa-binding protein for cholesterol absorption inhibitors in the enterocyte brush border membrane was identified as the ectopeptidase aminopeptidase N [17]. Recently, the Niemann–Pick 1 like 1 protein (NPC1L1) was described to be essential for intestinal cholesterol absorption [18]

Abbreviations: APN, Aminopeptidase N; BBMV, Brush border membrane vesicles; DTT, Dithiothreitol; PBS, Phosphate buffered saline

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and was suggested as the molecular target of ezetimibe [19]. According to a recent report, however, NPC1L1 does not reside in the plasma membrane but is localized intracellularly colocalizing with the small GTPase Rab 5 [20]. Since nonabsorbable ezetimibe analogues inhibit cholesterol absorption from the gut lumen without getting access to the enterocyte cytosol [16], the role of NPC1L1 as the primary target for ezetimibe is unclear due to it absence from the brush border membrane.

Smart and coworkers have demonstrated that in the zebrafish and the mouse, annexin II and caveolin 1 form in the cytosol a heteromeric protein complex resistant to boiling in SDS, reducing conditions and ether extraction [21]; ezetimibe was able to disrupt this heterocomplex by directly interacting with caveolin 1 and consequently the authors have suggested that the annexin II/caveolin 1 complex is a target of ezetimibe regulating intestinal cholesterol transport [21]. On the other hand, nonabsorbable ezetimibe analogues like AVE5530 or S6130, act exclusively from the intestinal lumen and do not reach the enterocyte cytosol, but are equipotent or even more potent than ezetimibe [17]. We therefore investigated whether an annexin II/caveolin 1 complex contributes to the molecular action of 2-azetidinone cholesterol absorption inhibitors in rabbit small intestine. In the present paper, we demonstrate that ezetimibe (analogues) neither bind directly to caveolin 1 nor annexin II nor that an annexin II/caveolin 1 heterocomplex exists in the brush border membrane or cytoplasm of rabbit small intestinal enterocytes thereby excluding such a complex as a primary target for ezetimibe.

2. Materials and methods

2.1. Materials

The cholesterol absorption inhibitors AVE 5530, ezetimibe, C-5, [^{3}H]C-2 (specific radioactivity 3.41 Ci/mmol), [^{3}H]ezetimibe (A_{sp}=21.102 Ci/mmol) and [^{3}H]C-6 (A_{sp}=1.22 Ci/mmol) (Fig. 6) were synthesized at Aventis Pharma Deutschland GmbH according to published procedures [12,13,22]. Triton X-100, acrylamide, *N*,*N*′-bismethylene acrylamide and Serva Blue R 250 were from Serva (Heidelberg, Germany). Marker proteins for the determination of molecular masses ("See Blue", "Multi Mark") were from Invitrogen (Karlsruhe, Germany). Nonidet P40 and deoxycholate were from Sigma (Munich, Germany). Streptavidin Sepharose and protein A-Sepharose were from Amersham Biosciences whereas scintillator Quickszint 501 and tissue solubilizer Biolute S were from Zinsser Analytic GmbH (Frankfurt, Germany). Protein was determined with a Bradford assay kit from Bio-Rad and enzymatic activities for aminopeptidase N were measured using the Merckotest Kit 3559 (Merck KgA, Darmstadt, Germany).

2.2. Antibodies

Antibodies against rabbit aminopeptidase N were prepared as described [17]. The following commercially available antibodies were used: Anti-actin (AC40, antibody 11003) from Abcam (Cambridge, UK), anti-annexin II (antibody 610069), and anti-caveolin 1 (antibody 610059) from BD Biosciences (Heidelberg, Germany), whereas the conjugate between streptavidin and alkaline phosphatase (RPN 1234) was from Amersham Biosciences.

2.3. Animals and membrane preparation

Male New Zealand White rabbits weighing 4–5 kg (Harlan Winkelmann, Borchem, Germany) were kept on Altromin® standard diet C 2023 (Altromin®, Lage, Germany) ad libitum. To investigate the effect of cholesterol absorption inhibitors on the composition of the BBMV, rabbits were treated for 4 days receiving once daily by gavage 2×3 mg ezetimibe or the nonabsorbable cholesterol absorption inhibitor AVE 5530 suspended in Tylose as vehicle or with vehicle alone. BBMV from rabbit jejunum and ileum were prepared as described [23] and cytosol was prepared by centrifugation of freshly prepared jejunum or ileum homogenate at 100 000×*g* for 60 min and was immediately used for experiments.

2.4. Immunoprecipitation

Ileal or jejunal BBMV (60–100 μg of protein) were solubilized at 1 mg/ml in "Lysis"-buffer [21] (50 mM Tris–HCl buffer (pH 7.2)/150 mM NaCl/1% Triton X–100/1% deoxycholate/0.1% SDS) at 20 °C for 60 min followed by centrifugation. Immunoprecipitation experiments were performed as follows: solubilized BBMV-proteins in Lysis-buffer (60–100 μg of protein, 1 mg/ml) or freshly prepared jejunal or ileal cytosol (either in phosphate buffered saline or lysis buffer, 50–100 μg at 1 mg/ml) were incubated with anti-annexin II or anti-caveolin antibodies (usually 2 μg) and incubated for 12 h at 4 °C. Subsequently, 10 μl of protein A-beads – suspended in lysis-buffer/30 mg/ml BSA – were added and the samples were kept at 4 °C under stirring for 2 h. After centrifugation, the supernatants were removed for analysis by SDS-PAGE and the beads were washed five times with 50 mM Tris–HCl buffer (pH 7.2)/200 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS. The beads were subsequently immersed in SDS-sample buffer (62.5 mM Tris/HCl (pH 6.8)/2% SDS/100 mM DTT/10% glycerol/0.001% bromophenol blue) and after centrifugation, the supernatants were analysed by SDS-PAGE.

2.5. Binding of [^{3}H]labelled ezetimibe analogues to protein components of rabbit jejunal or ileal cytosol or BBMV

In order to detect a specific binding of ezetimibe analogues to caveolin, jejunal or ileal cytosol (40 μg of protein) at 1 mg/ml in Tris/HCl buffer (pH 7.4)/300 mM mannitol were incubated with 0.63 μCi [^{3}H]ezetimibe or 1 μCi of [^{3}H]C-6 for 2 h at 30 °C. Afterwards, 2 μg of anti-caveolin 1 antibodies in 20 μl of 10 mM Tris/HCl-buffer (pH 7.4)/300 mM mannitol were added and the samples were kept at 4 °C for 12 h. BBMV (25–40 μg of proteins) were incubated for 2 h with the [^{3}H]labelled ezetimibe-derivatives; after dilution with 350 μl of PBS, membranes were collected by centrifugation and solubilized in 100 μl of Lysis-buffer after 1 h at 4 °C. After centrifugation 2 μg of anti-caveolin 1 antibodies in 20 μl of 10 mM Tris/HCl buffer (pH 7.4)/300 mM mannitol were added to the clear supernatant containing solubilized BBMV protein and the solution was kept at 4 °C for 12 h. Afterwards, protein A-Sepharose-beads (10 μl) were added and the immunoprecipitation was performed as described above. Aliquots from each step including wash-steps were counted for radioactivity.

2.6. Photoaffinity labelling

BBMV from rabbit jejunum or ileum (100–150 μg of protein) were incubated at 1 mg/ml with [^{3}H]labelled ezetimibe analogues in 10 mM Tris/HCl buffer (pH 7.4)/100 mM NaCl/100 mM mannitol for 60 min at 20°C in the dark followed by ultraviolet radiation in a Rayonet RPR-100 photochemical reactor equipped with 4 RPR 2543-Å lamps for 120 s (C-2, C-6, ezetimibe). For labelling with the biotin-tagged probe C-5, 10–30 μg of protein was used and irradiation at 254 nm was performed for 30 s Afterwards, BBMV were diluted with 10 mM Tris/HCl buffer (pH 7.4)/300 mM mannitol/1 mM Pefabloc and washed with this buffer 3 times followed by SDS-PAGE. For immunodetection of labelled proteins, jejunal or ileal BBMV were photolabeled with [^{3}H]C-2, [^{3}H]ezetimibe or [^{3}H]C-6 followed by SDS-PAGE and blotting; after immunostaining with anti-annexin II, anti-caveolin 1, anti actin or anti-APN antibodies, the immunoreactive bands were marked and afterwards the blots were sliced into 2 mm pieces, dissolved in tissue solubilizer Biolute S and radioactivity was determined by liquid scintillation counting.

2.7. SDS Gel electrophoresis

SDS-PAGE was carried out in vertical stab gels (20×17×0.15 cm) using an electrophoresis system LE 2/4 (Amersham Pharmacia Biotech, Freiburg, Germany) with gel concentrations of 7–10.5% at a ratio of 97.2% acrylamide

and 2.8% *N*,*N*-methylene bisacrylamide or in pre-casted NOVEX gels (4–12%, 12% or 15%, Invitrogen, Groningen, The Netherlands) using an electrophoresis system XCell II from Novex. After electrophoresis the gels were fixed in 12.5% trichloroacetic acid followed by staining with Serva Blue R 250. For determination of the distribution of radioactivity, individual gel lanes were cut into 2 mm pieces, protein was hydrolyzed with 250 µl of tissue solubilizer Biolute S and after addition of 4 ml of scintillator Quickszint 501 radioactivity was measured by liquid scintillation counting. Western blotting and immunostaining was performed as described earlier [24].

3. Results

3.1. Immunoprecipitation with annexin II and caveolin 1-antibodies from rabbit small intestinal BBMV and cytosol

In order to detect a heteromeric protein complex between annexin II and caveolin 1 in the rabbit, as was found in the zebrafish and the mouse, freshly prepared BBMV as well as cytosol from rabbit jejunum and ileum were analysed by Western blotting. Fig. 1a demonstrates that in the BBMV from rabbit jejunocytes and ileocytes (data not shown), annexin II, caveolin 1, actin as well as aminopeptidase N were detectable as protein bands of M_r 36 kDa (annexin II), 20 and 22 kDa

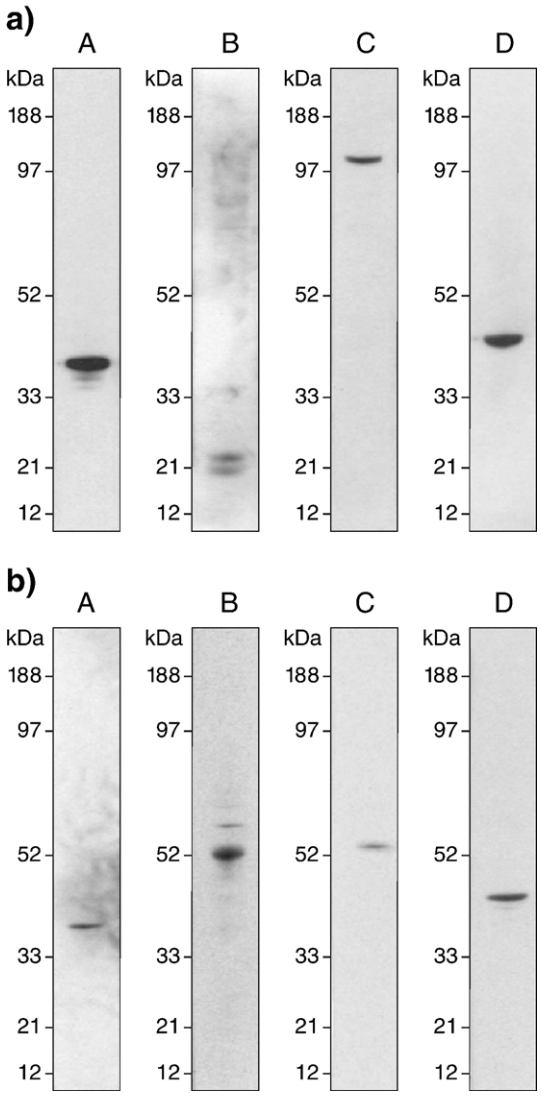


Fig. 1. Immunoblotting of cytosol and BBMV from rabbit jejunum. 5 µg of protein of freshly prepared BBMV (a) or cytosol (b) from rabbit jejunum were separated by SDS-PAGE followed by blotting and immunostaining with the respective antibodies: (A) Anti-annexin II; (B) Anti-caveolin-1; (C) Anti-APN; (D) Anti-actin.

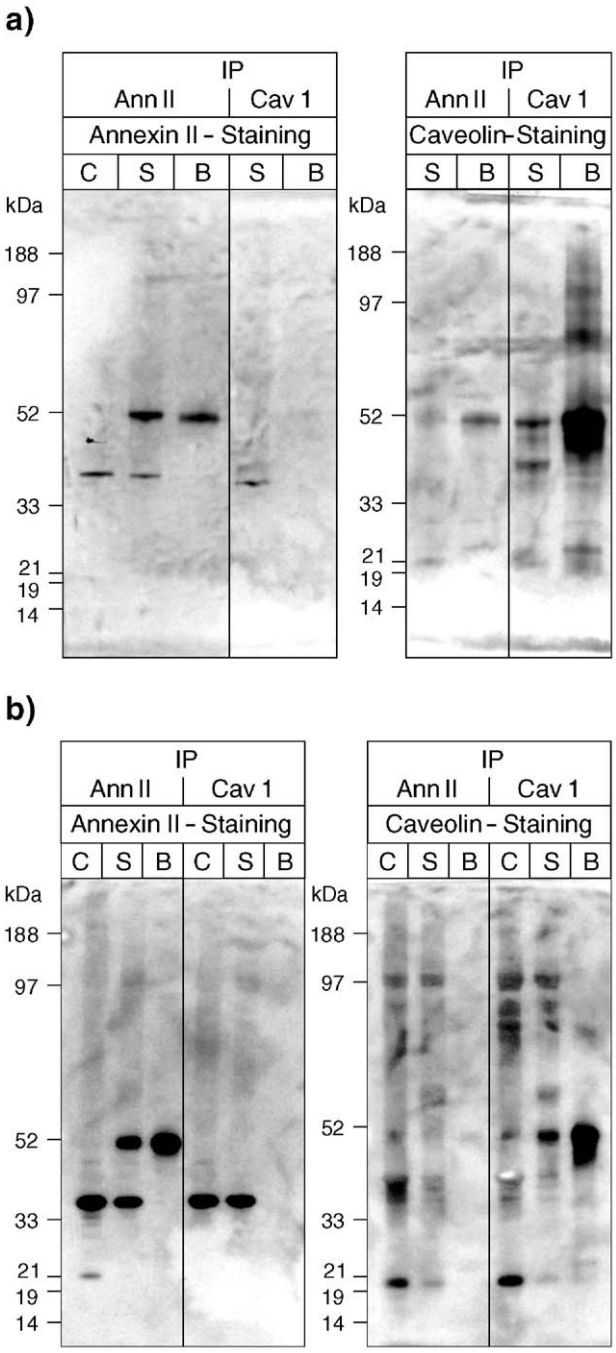


Fig. 2. Immunoprecipitation with anti-annexin II and anti-caveolin 1 antibodies from cytosol or solubilized BBMV from rabbit jejunum. To 60 µg of freshly prepared cytosol (a) or solubilized BBMV (b) from rabbit jejunum (1 mg/ml), 2 µg of either anti-annexin II or caveolin 1-antibodies were added followed by addition of protein A Sepharose beads after 12 h of incubation. After washing, proteins were extracted from the beads with SDS-sample buffer followed by SDS-PAGE and immunostaining: C: Control cytosol or BBMV; S: Supernatants after 12 h of antibody addition; B: Extracts from protein A sepharose beads.

(caveolin 1), 43 kDa (actin) and 145 kDa (APN) indicating their presence as monomeric proteins on the SDS-gels; no indications for a heteromeric complex of M_r 52 kDa for annexin II/caveolin 1 could be found independently on the membrane preparation (ten different membrane preparations with the identical isolation protocol) and the conditions of sample preparation for SDS-PAGE. Western blot analysis of freshly prepared cytosol (Fig. 1b) from rabbit jejunum or ileum likewise exclusively revealed bands for the monomeric forms of annexin II (36 kDa, lane A) or actin (43 kDa, lane D), whereas neither a 20-kDa caveolin 1 band (lane B) nor a 145-kDa APN band (lane C) could be detected in the cytosol. After immunostaining. with anti-caveolin-1 antibodies (Fig. 1b, lane B) or anti-APN-antibodies (Fig. 1b, lane C), however, a 52-kDa band was detectable in the cytosol (Fig. 1b, lane C). We therefore performed immunoprecipitation experiments with caveolin 1 and annexin II antibodies using exactly the conditions described by Smart et al. [21]. Fig. 2a shows that after immunoprecipitation of rabbit jejunal cytosol with annexin II antibodies a 52-kDa band immunoreactive with both annexin II and caveolin 1 antibodies was detectable; after immunoprecipitation with caveolin-1 antibodies, a very strong 52 kDa band was detectable with caveolin 1 antibodies, whereas with annexin II antibodies no 52 kDa band could be detected in the extract from the protein A beads. If rabbit jejunal (or ileal) BBMV were solubilized and the solubilized membrane proteins submitted to the identical protocol, similar results were obtained (Fig. 2b); after immunoprecipitation with annexin II antibodies, a strong annexin II-immunoreactive band of 52 kDa was observed in the protein-A-bead extract, whereas only a faint 52 kDa-band was seen after staining with caveolin-1 antibodies. Vice versa, immunoprecipitation with caveolin 1 antibodies produced a very strong 52 kDa caveolin 1- positive band whereas no staining of a 52-kDa band was detectable with annexin II antibodies. It was reported that in the zebra fish, in LDL-receptor-mutant mice or in mice fed a

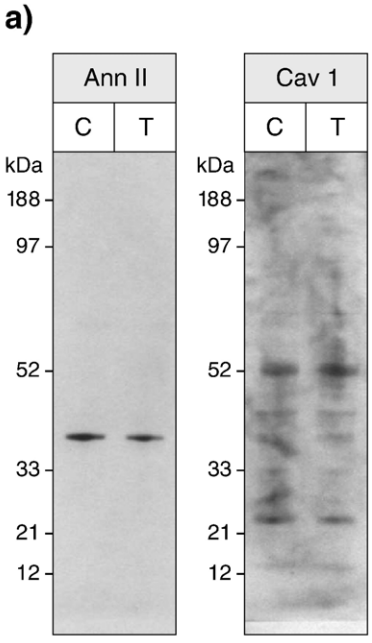


Fig. 3. Immunostaining of cytosol or BBMV of rabbit jejunum after treatment of rabbits with the nonabsorbable cholesterol absorption inhibitor AVE 5530. Cytosol (a) and BBMV (b) from control (C) or animals treated with the nonabsorbable cholesterol absorption inhibitor AVE 5530 (T) were freshly prepared and 5 μg of protein per lane were analysed by SDS-PAGE and subsequent immunostaining with the indicated antibodies.

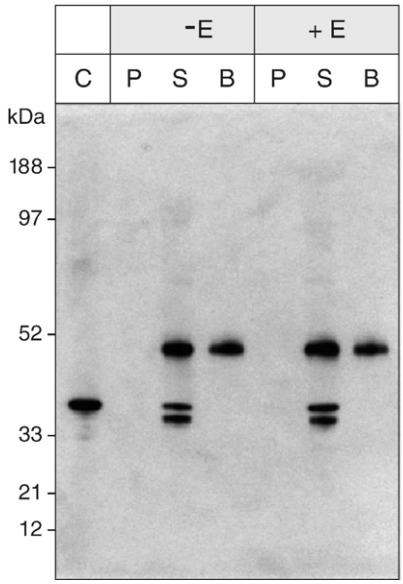
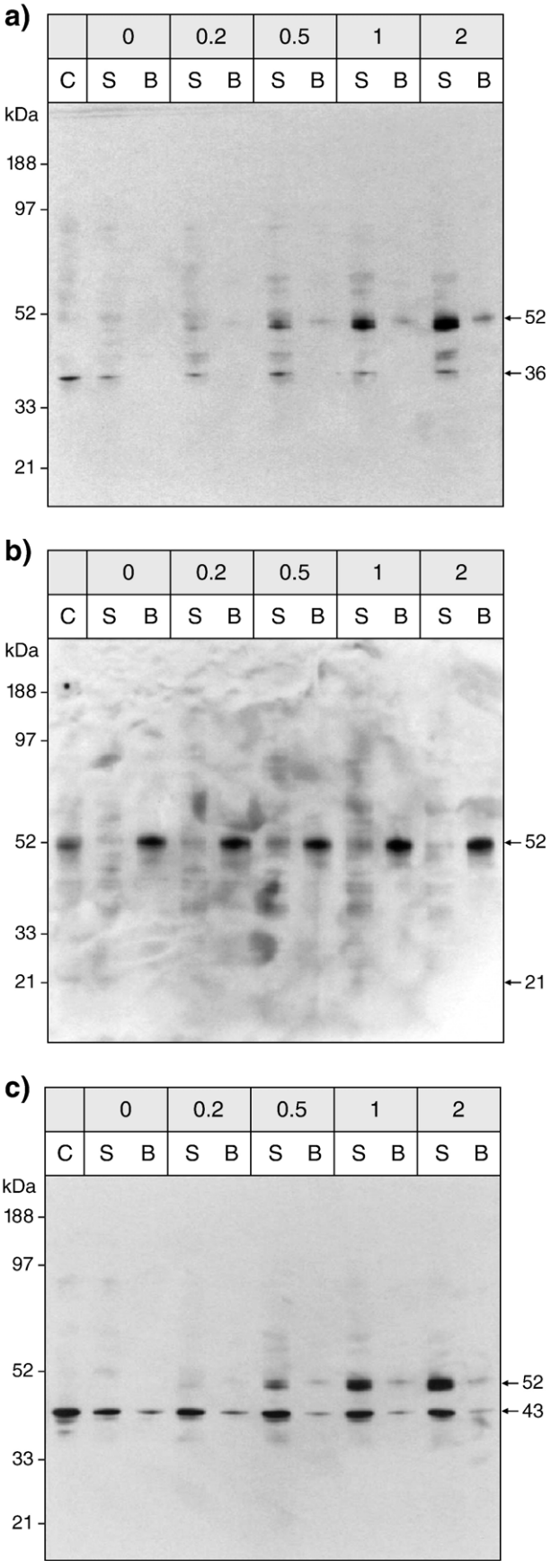


Fig. 4. Effect of ezetimibe on the 52-kDa band after immunoprecipitation with anti-annexin II antibodies from rabbit jejunal BBMV. 40 μg of rabbit jejunal BBMV in 10 mM Tris/HCl buffer (pH 7.4)/300 mM mannitol were incubated without (−E) or with 250 μM ezetimibe (+E) for 4 h at 20 °C. Subsequently, BBMV were solubilized and immunoprecipitation was performed as described in the experimental section with 3 μg of anti-annexin II antibodies followed by SDS-PAGE and immunostaining. C: Control BBMV; P: Pellet after solubilization; S: Supernatant after 12 h of antibody addition; B: Extract from protein A sepharose beads.



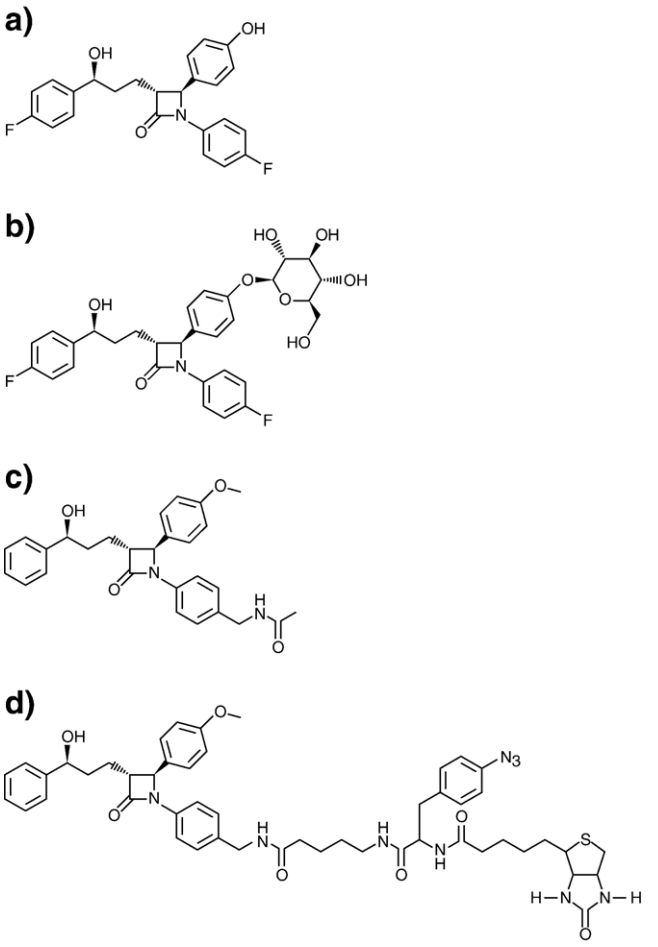


Fig. 6. Structures of 2-azetidinone cholesterol absorption inhibitors. (a) Ezetimibe; (b) Glycoside C-6; (c) Photoaffinity probe C-2; (d) Biotin-tagged photoaffinity probe C-5.

hypercholesterolemic diet, ezetimibe disrupts the annexin II/caveolin 1 complex [21]. Treatment of rabbits with ezetimibe or the nonabsorbable highly potent cholesterol absorption inhibitor AVE 5530 had no effect on the putative formation or dissociation of an annexin II/caveolin 1-complex; in jejunal and ileal cytosol or BBMV of untreated or drug-treated animals, identical western blot patterns were observed with annexin II as a 36-kDa monomer, whereas with caveolin 1 antibodies, 20 and 22 kDa bands occurred (Fig. 3). In addition, incubation of jejunal cytosol or rabbit jejunal or ileal BBMV with ezetimibe or AVE 5530 and subsequent immunoprecipitation with annexin II or caveolin 1-antibodies (after solubilization of BBMV) had no effect on the

Fig. 5. Influence of the amount of antibody on the intensity of the 52-kDa band. Freshly prepared rabbit jejunal cytosol (40 μg of protein) were completed with 0, 0.2, 0.5, 1 or 2 μg of anti-annexin II antibodies followed by extraction with protein A–sepharose beads. Aliquots of the supernatants and the protein-A-beads extracts were analysed by SDS-PAGE and immunostaining with anti-annexin II, anti-caveolin 1, anti-actin and anti-APN antibodies. C: Control cytosol; S: Supernatant after 12 h of antibody incubation; B: Extract from protein A-sepharose beads. (a) After staining with anti-annexin II-antibodies; (b) after staining with anti-caveolin 1-antibodies; (c) after staining with anti-actin-antibodies.

Table 1
Binding of [^{3}H]labelled ezetimibe analogues to caveolin 1 after immunoprecipitation with anti-caveolin 1 antibodies from cytosol and BBMV from rabbit jejunum or ileum

Protein sample	Incubation		Bound to protein A beads extracts		
	Amount (fmol)	Incubated (dpm, μCi)	fmol	dpm	% of applied amount
[^{3}H]Ezetimibe					
Cytosol-jejunum	15.85×10^3	8.658×10^5 (0.39)	3.51	157	0.022
	13.78×10^3	7.67×10^5 (0.345)	1.41	66	0.01
Cytosol-ileum	15.02×10^3	8.70×10^5 (0.392)	3.56	168	0.023
BBMV-ileum	10.62×10^3	8.97×10^5 (0.404)	1.58	74	0.015
	2.16×10^3	1.01×10^5 (0.045)	0.32	15	0.028
[^{3}H]-C-6					
Cytosol-jejunum	664.06	2.077×10^6 (0.935)	<53.5	145	0.008
BBMV-ileum	258.6	8.48×10^5 (0.382)	<8.48	23	0.0033

Freshly prepared cytosol or BBMV from rabbit jejunum or ileum were incubated with [^{3}H]labelled ezetimibe analogues followed by immunoprecipitation with anti-caveolin 1 antibodies.

polypeptide pattern after staining with annexin II or caveolin 1-antibodies (Fig. 4).

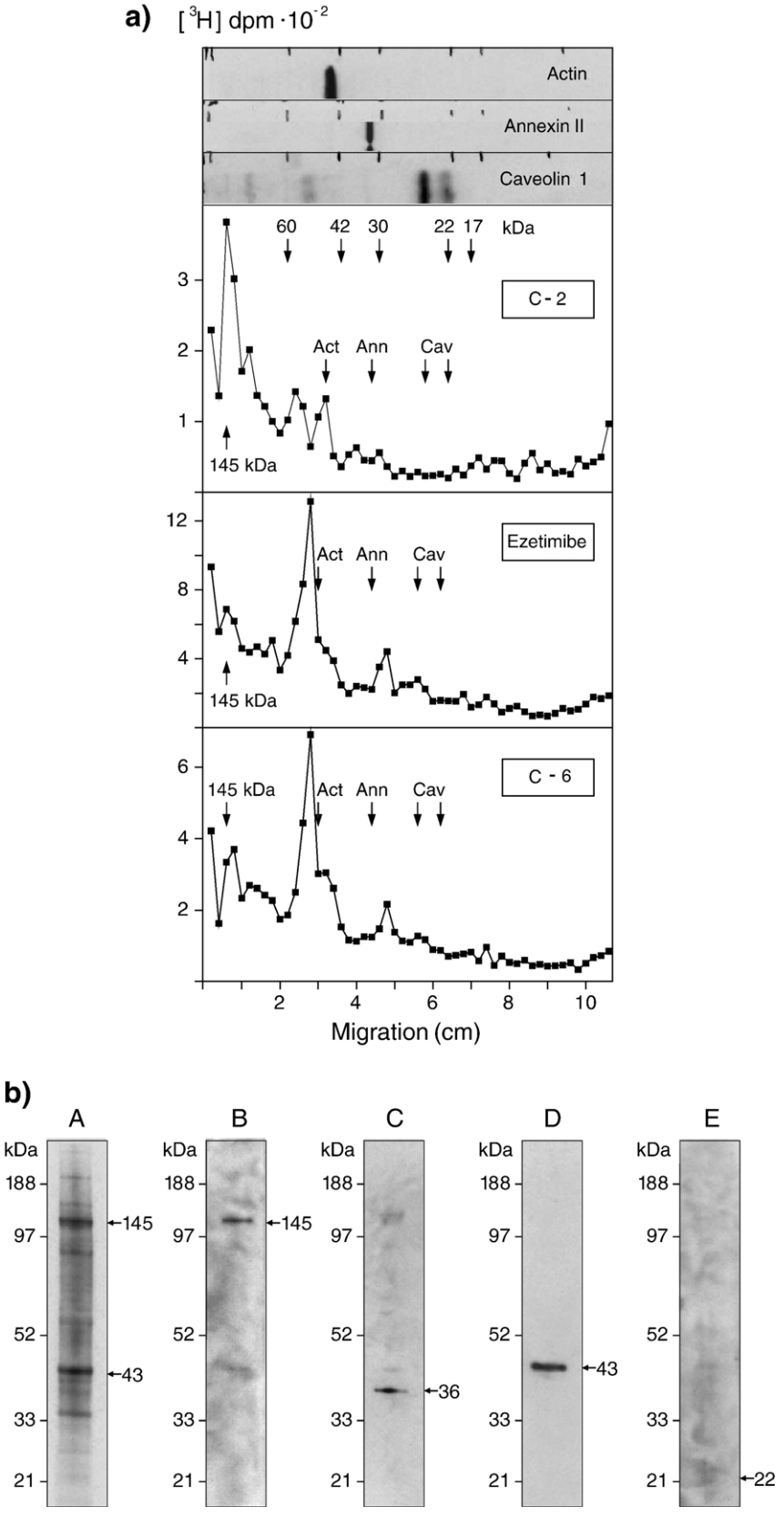
Smart et al. reported that in the mouse annexin II forms a heteromeric 52 kDa complex with caveolin 1 in the small intestine whereas in mouse aorta annexin II occurs as a monomer (21) offering the possibility that annexin II and caveolin 1 may occur in different organs or organelles either as monomers or as heterodimers. Western blot analysis of homogenates from rabbit jejunum or rabbit liver, rabbit jejunal BBMV and cytosol from small enterocytes revealed in any case, that caveolin 1 an annexin II exist in the rabbit exclusively as monomers; after heating of the samples in SDS-sample buffer, a 52-kDa band occurred after caveolin 1 staining, but not after annexin II-staining excluding the possibility that a 52-kDa heterocomplex between annexin II and caveolin 1 is formed by the procedure of sample preparation. The findings that with antibodies against caveolin 1 and APN, a 52-kDa band was detectable in rabbit intestinal cytosol and that by immunoprecipitation with anti-caveolin-1 antibodies from cytosol and solubilized BBMV a caveolin 1-immunoreactive 52 kDa band was detectable in the protein-A bead extracts lacking immunoreactivity against annexin II suggest the possibility of a putative artefact for the 52-kDa band. Fig. 5a shows that the amount of the 52-kDa anti-annexin II-positive band increased with the amount of annexin II-antibodies added; in the cytosol control (Fig. 5a, lane C) no 52 kDa band could be observed, whereas with increasing amounts of anti annexin II antibodies, the 52-kDa band appeared in the protein A sepharose extract (lanes B) and the cytosol-supernatant (lanes S). Similarly, the amount of a 52-kDa band, immunoreactive with caveolin 1 (Fig. 5b) and actin (Fig. 5c) showed a clear dependency on the amount of antibody used for immunoprecipitation. Analogous results were obtained with solubilized BBMV from rabbit jejunum or ileum. In control experiments where phosphate buffered saline was used instead of cytosol or BBMV, a 52-kDa band was also stained in the protein A extracts with annexin II or caveolin 1 antibodies after "immunoprecipitation" with annexin II or caveolin 1 antibodies demonstrating that the 52-kDa band was caused by the added antibodies.

3.2. Interaction of ezetimibe (analogues) with annexin II and caveolin 1

Since it was suggested that ezetimibe disrupts the annexin II/caveolin 1 complex most likely through a direct interaction with caveolin 1 [21], we investigated the binding of radiolabeled ezetimibe and ezetimibe analogues to proteins of the brush border membrane of rabbit small intestinal enterocytes. Either freshly prepared cytosol from rabbit jejunocytes or ileocytes or jejunal or ileal BBMV were incubated with radioactively labelled ezetimibe or the ezetimibe glucoside C-6 (Fig. 6); BBMV were subsequently solubilized and the solubilized BBM-proteins or cytosol were subjected to immunoprecipitation with caveolin 1 antibodies exactly as described [21] followed by determination of radioactivity in the immunoprecipitates. Table 1 demonstrates that neither for ezetimibe nor for the nonabsorbable glucoside C-6 a significant binding to the caveolin 1-immunoprecipitate was detectable; after incubation with 2–15 pmol of [^{3}H]ezetimibe (0.3–1 μCi) 0.3–3.6 fmol (15–168 dpm) were detectable in the caveolin 1 immunoprecipitate despite the high specific radioactivity of 21.1 Ci/mmol of the probe; with the glucoside C-6 less than 0.008% of the applicated radioactivity was found in the precipitate at the threshold of background activity. Furthermore, indistinguishable low counts were found by immunoprecipitation using

Fig. 7. Photoaffinity labelling of rabbit jejunal BBMV with ezetimibe-derivatives. Rabbit jejunal BBMV (200 μg of protein) were incubated at 1 mg/ml in 10 mM Tris/HCl buffer (pH 7.4)/100 mM NaCl/100 mM mannitol with 4.88 μM (2.5 μCi) [^{3}H]C-2, 0.79 μM (2 μCi) [^{3}H]ezetimibe or 13.66 μM (2 μCi) [^{3}H]C-6 for 60 min at 20 °C followed by ultraviolet irradiation at 254 nm for 120 s After washing, proteins were separated by SDS-PAGE and after blotting and immunostaining, the nitrocellulose sheets were cut into 2 mm pieces for determination of the distribution of radioactivity. Rabbit jejunal BBMV (200 μg of protein) were photolabeled with 10 μM of the biotin-tagged photoaffinity probe C-5 and after washing and solubilization proteins were extracted with streptavidin beads [17]. The extracts were analysed after SDS-PAGE by immunostaining with streptavidin-alkaline phosphatase conjugate for C-5 labelled proteins (A) and with antibodies against APN (B), annexin II (C), actin (D) and caveolin 1 (E).

annexin II antibodies instead of caveolin-1 antibodies. Control experiments were performed with solubilized BBMV-proteins without addition of caveolin 1 antibodies but with otherwise identical protocol. In the immunoprecipitates (protein A bead extract) indistinguishable low counts of [^{3}H]ezetimibe were found as after addition of caveolin 1 antibodies. In contrast,



strong immunoreactive bands for caveolin 1 were detectable only in the bead extracts after addition of caveolin 1-antibodies. This finding clearly shows that the low amounts of [^{3}H] ezetimibe found in the protein A-bead extracts were independently on the presence of caveolin 1. Thus, these results do not give any evidence for a specific binding of ezetimibe (analogues) to caveolin 1.

To demonstrate a putative direct binding of ezetimibe to caveolin 1 or annexin II we have performed photoaffinity labelling studies using the radiolabelled ezetimibe analogues C-2, ezetimibe, the ezetimibe glycoside C-6 as well as the biotin-tagged ezetimibe photoaffinity probe C-5 (Fig. 6). The suitability of ezetimibe, C-2 and ezetimibe glycoside C-6 for direct photoaffinity labelling was demonstrated by a photocatalysed covalent incorporation into albumin and small intestinal BBMV upon ultraviolet irradiation at 254 nm for 90 to 120 s using the identical setup of control experiments as for the direct photoaffinity probe [^{3}H]benzylpenicillin [25]. Fig. 7a shows that the photoaffinity probe C-2 predominantly incorporated into proteins of M_r 145 kDa, 98 kDa, 62 kDa and 43 kDa as described earlier [11]. Ezetimibe and the glucoside C-6 showed the highest incorporation into 56 kDa proteins and the 145-kDa protein with a weak labelling of a 28-kDa band. Western blotting revealed that none of the radioactively labelled bands obtained with the different ezetimibe-analogues did co-migrate with annexin II or caveolin 1. In contrast, the 43-kDa band co-migrated with actin and the 145-kDa band with aminopeptidase N and the identity of these radiolabeled proteins with actin and APN was demonstrated by sequence analysis [17]. Additionally, after photoaffinity labelling with radiolabeled ezetimibe photoaffinity probes and subsequent solubilization, no radioactively labelled proteins could be precipitated with antibodies against annexin II or caveolin 1 (data not shown). It maybe argued, that the lack to detect a radiolabeled caveolin 1 or annexin II band is caused by a change in immunoreactivity of the anti-caveolin 1 or anti-annexin II antibodies to caveolin 1 and annexin II upon binding of ezetimibe to these proteins. Dot blot analysis of BBMV, cytosol or jejunum homogenate being incubated with 100 μM ezetimibe did not reveal any change of immunoreactivity of caveolin 1 or annexin II antibodies by the presence of ezetimibe nor was the immunoreactivity changed in Western blotting where ezetimibe was present throughout the renaturating, blocking and immunostaining process. Rat adipocyte membranes contain considerable amounts of caveolin; photoaffinity labelling of rat adipocyte membranes with radioactively labelled C1 and C-2 or the biotin-tagged C-5 ezetimibe analogues revealed after SDS-PAGE very strong immunoreactive 19 and 21 kDa bands for caveolin 1 but no comigration or superposition of these bands with any bands labelled by the ezetimibe analogues. The biotin-tagged ezetimibe photoaffinity probe C-5 can be used to isolate ezetimibe-binding proteins from rabbit small intestinal BBMV after photoaffinity labelling and solubilization by streptavidin–biotin affinity extraction as demonstrated elsewhere [13,17]. The purified material showed a prominent labelling of a 145-kDa and a 43-kDa protein by the ezetimibe analogue C-5 (Fig. 7b, lane A), which cross-react with anti-APN-antibodies (Fig. 7b, lane B) and anti actin-antibodies (Fig. 7b, lane D). Immunostaining with anti-annexin II antibodies (Fig. 7b, lane C) revealed the presence of annexin II in the purified material, whereas with anti-caveolin 1-antibodies no caveolin 1 - protein was detectable in the streptavidin-purified material (Fig. 7b, lane E). However, no C-5-labelled 36 kDa (annexin II) and 20/22 kDa (caveolin 1) bands could be detected in the streptavidin-extract after photoaffinity labelling of rabbit jejunal BBMV with the biotin-tagged ezetimibe photoaffinity probe C-5 (Fig. 7b, lane A). In summary, these series of experiments clearly demonstrate that ezetimibe or ezetimibe analogues do not directly and specifically bind to annexin II and caveolin 1.

4. Discussion

Cholesterol absorption involves formation of mixed micelles containing cholesterol, bile acids, fatty acids and phospholipids followed by protein-catalysed release of monomeric cholesterol [17,26], which is distributed into the brush border membrane and moved to detergent-resistant microdomains from which transport to the endoplasmic reticulum occurs [27]. By photoaffinity labelling with photoreactive analogues of the cholesterol absorption inhibitor ezetimibe, we identified a 145-kDa membrane protein – sequenced as aminopeptidase N (CD 13) – as the primary target for cholesterol absorption inhibitors in the enterocyte brush border membrane [11,15–17]. We could demonstrate that incubation of confluent $CaCo_2$ cells with ezetimibe led to a strong decrease of APN-expression of the plasma membrane suggesting that binding of ezetimibe to APN interferes with the cellular trafficking of APN between the plasma membrane and intracellular compartments [17]. The use of nonabsorbable ezetimibe analogues proved that binding of cholesterol absorption inhibitors from the lumen to APN is sufficient to inhibit cholesterol absorption, i.e. an intracellular target for ezetimibe (analogues) is not necessary to exert its (their) primary pharmacological effect to block intestinal cholesterol absorption. It is however not yet known how the binding of cholesterol absorption inhibitors interferes with cholesterol trafficking from the brush border membrane to intracellular compartments. Annexins are a family of caveolin- and phospholipid-binding proteins with an involvement in a variety of cellular processes like membrane fusion, vesicle transport or channel formation [28]. Annexin II is assumed to be involved in endocytosis and secretion [29–31], and it was demonstrated that annexin II is required for the biogenesis of multivesicular transport intermediates destined for late endosomes [32]. Annexin II interacts with cholesterol and its subcellular distribution is modulated by the cholesterol distribution, and it was suggested that annexin II forms cholesterol-containing platforms, which regulate the onset of the degradation pathways in animal cells [32].

A role of annexin II for intestinal cholesterol absorption was recently reported by Smart et al. [21]; they describe a SDS-resistant heterocomplex formed by annexin II and caveolin 1 in the cytosol of the intestine of the zebra fish and partially in the mouse regulating intestinal cholesterol transport leading the authors to conclude “that this SDS-

resistant heterocomplex is a general feature likely to be present in all vertebrates". In mouse aorta however, caveolin 1 and annexin II were exclusively found as monomers [21]. In enterocytes from rabbit jejunum or ileum however, annexin II and caveolin 1 were exclusively found as monomers; under no circumstances a 52-kDa heterocomplex between annexin II and caveolin 1 could be found. In addition, after treatment of rabbits with pharmacologically active doses of ezetimibe or nonabsorbable ezetimibe analogues like AVE 5530 no changes in the aggregation of caveolin 1 or annexin II occurred: caveolin 1 and annexin II were detectable exclusively as monomers indicating the absence of a heteromeric annexin II / caveolin 1-complex in rabbit small intestine. If, however, cytosol or solubilized BBMV-proteins from rabbit jejunum or ileum were submitted to the immunoprecipitation protocol as described [21], a 52-kDa band occurred in addition to the monomers after addition of caveolin 1 or annexin II antibodies. After extraction with protein A-sepharose beads, immunoreactive 52 kDa bands were observable after immunoprecipitation with annexin II and subsequent annexin II-staining (Fig. 2); however, no 52 kDa staining occurred with caveolin 1-antibodies. Vice versa, after immunoprecipitation with caveolin 1-antibodies, a strong 52 kDa caveolin 1-positive band was seen whereas annexin II-staining did not reveal a 52-kDa band. These findings suggest that the 52-kDa band is produced by addition of the antibodies and the intensity of the 52-kDa band increased with the amount of antibody added. Furthermore, after annexin II – immunoprecipitation, staining with antibodies against actin or aminopeptidase N, also a 52-kDa band was detectable. These findings indicate that in the rabbit the 52-kDa band obtained by immunoprecipitation with annexin II or caveolin 1-antibodies is a biochemical artefact, probably produced by staining of the heavy chain of the IgG-immunoglobulins by unspecific binding of the respective antibody-alkaline phosphatase conjugates. After incubation of rabbit intestinal cytosol or BBMV with radiolabeled ezetimibe or an ezetimibe glucoside no co-precipitation with caveolin 1 was found indicating that ezetimibe derivatives do not directly bind to caveolin 1. After photoaffinity labelling of rabbit small intestinal BBMV or cytosol with different radiolabeled ezetimibe analogues, no incorporation of the ezetimibe derivatives into proteins of M_r 36 kDa or 20/22 kDa was observed nor could labelled proteins of these molecular masses been immunoprecipitated with antibodies against annexin II or caveolin 1. In contrast, photoincorporation of the ezetimibe analogues occurred into the 145-kDa and 43-kDa proteins which were identified as aminopeptidase N and actin, respectively [17]. From these findings, we conclude that in the rabbit neither annexin II or caveolin 1 nor a heteromeric annexin II–caveolin 1 complex are direct molecular targets for cholesterol absorption inhibitors like ezetimibe. A paper published during preparation of this manuscript showed that caveolin-1 is not required for murine intestinal cholesterol transport also questioning a role of caveolin-1 as a primary target for ezetimibe [33]. It remains to be elucidated how binding of ezetimibe to its primary target APN in the enterocyte brush border membrane [17] interferes with the annexin II-regulated biogenesis of multivesicular endosomes destined for late endosomes.

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