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Function of MRP1 is not dependent on cholesterol or cholesterol-stabilized lipid rafts

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Synopsis

MRP1[‡] has been localized in cholesterol-enriched lipid rafts, which suggests a role for these lipid rafts and/or cholesterol in MRP1 function. Here we show for the first time that nearly complete oxidation of free cholesterol in the plasma membrane of BHK-MRP1 cells did not affect MRP1 localization in lipid rafts or its efflux function, using 5-carboxyfluorescein diacetate as substrate. Inhibition of cholesterol biosynthesis, using lovastatin in combination with RO 48-8071, resulted in a shift of Mrp1 out of lipid raft fractions, but did not affect Mrp1-mediated efflux in Neuro-2a cells. Short-term methyl- β -cyclodextrin treatment was equally effective in removing free cholesterol from Neuro-2a and BHK-MRP1 cells, but affected MRP1 function only in the latter. The kinetics of loss of both MRP1/Mrp1 efflux function and lipid raft association during long-term methyl- β -cyclodextrin treatment did not match the kinetics of free cholesterol removal in both cell lines. Moreover, MRP1 activity was measured in vesicles consisting of membranes isolated from BHK-MRP1 cells using the substrate cysteinyl leukotriene C₄ and was not changed when the free cholesterol level of these membranes was either decreased or increased. In conclusion, MRP1/Mrp1 activity is not correlated with the level of free cholesterol or with localization in cholesterol-dependent lipid rafts.

Page heading title: MRP1[‡] function is independent of cholesterol

Supplementary Key Words: methyl- β -cyclodextrin, lovastatin, RO 48-8071, cholesterol oxidase, ATPase activity; LTC₄ transport assay

[‡]Abbreviations: ABC, ATP-binding cassette; CDC, methyl- β -cyclodextrin/cholesterol; CFDA, 5-carboxyfluorescein diacetate; CO, cholesterol oxidase; DRM, detergent resistant membrane; LO, lovastatin; LTC₄, cysteinyl leukotriene C₄; M- β -CD or CD, methyl- β -cyclodextrin; MRP1, multidrug-resistance-related protein 1; RO, RO 48-8071

Introduction

One of the best characterized multidrug resistance mechanisms is the energy-dependent drug efflux by proteins belonging to the ATP-binding cassette (ABC) transporter protein superfamily. Multidrug-resistance-related protein 1 (MRP1) and P-glycoprotein are the most widely studied ABC transporters and are known to depend on their direct lipid environment for optimal functioning [1,2]. Lavie et al. [3] have shown for the first time the association of an ABC transporter with a membrane domain. They found that a substantial fraction of P-glycoprotein was located in Cav-1 containing Triton X-100-based detergent resistant membranes (DRMs) in P-glycoprotein overexpressing cells. Later studies showed localization of both P-glycoprotein and MRP1 in non-caveolar DRMs. Both ABC transporters were more strongly enriched in Lubrol-based or Brij-based DRMs compared to Triton X-100-based DRMs [4,5].

Given their localization in DRMs, the function of ABC transporters may well be dependent on cholesterol, which is known to be enriched in DRMs. Modulation of P-glycoprotein function by cholesterol and involvement of DRMs in this process are widely studied [6]. For example, cholesterol depletion resulted in a shift of P-glycoprotein out of DRM fractions and P-glycoprotein-mediated drug transport was also affected [7]. In Caco-2 cell monolayers, cholesterol depletion significantly impaired the efflux activity of P-glycoprotein [8]. Concerning modulation of MRP1 function by cholesterol, information is scarce. In one study, cholesterol was reported to modulate MRP1 function and this was related to the presence of MRP1 in DRMs [9]. It is important to rigorously establish whether cholesterol affects MRP1 function, as this could be the underlying mechanism for the appearance of MRP1 in lipid rafts and a potential target for manipulation of MRP1 activity in the context of tumour cell sensitization to cytostatics.

In this study we rigorously investigated the impact of cholesterol and the localization of MRP1/Mrp1 in cholesterol-dependent lipid rafts on efflux function of the ABC transporter in two cell lines. We used Neuro-2a (neuroblastoma) cells, which express endogenous murine Mrp1/Abcc1 and BHK-MRP1 (fibroblast) cells, which highly express stable transfected human MRP1/ABCC1 [10]. In addition, we studied ABCC1 function in vesicles consisting of membranes isolated from cholesterol-modulated BHK-MRP1 cells. In view of potential drawbacks using detergent-based protocols for lipid raft isolation [11,12], we employed the detergent-free lipid raft isolation procedure recently developed by Macdonald and Pike [13]. Various strategies were employed to manipulate cellular cholesterol levels, including 1) short- and long-term methyl- β -cyclodextrin (M- β -CD) treatment, which physically removes cholesterol from the plasma membranes of cells, 2) cholesterol oxidase treatment, which chemically converts cholesterol into cholestenon and 3) combined treatment with lovastatin, an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase and RO 48-8071, an inhibitor of oxidosqualene cyclase. Abcc1/ABCC1 activity did not correlate with the variable levels of cholesterol, either in intact cells or isolated membranes. Neither did Abcc1/ABCC1 activity correlate with the protein's variable extent of lipid raft localization resulting from cholesterol modulation. We conclude that Abcc1/ABCC1 function is not dependent on cholesterol or cholesterol-dependent lipid raft localization, while the observed effect of (long-term) M- β -CD treatment in intact cells, likely relates to reduced membrane integrity.

Materials and methods

Materials

MK571 was a gift from Prof. A.W. Ford-Hutchinson (Merck-Frosst, Inc., Kirkland, Canada). All cell culture plastic was from Costar (Cambridge, MA, USA). Cell culture media, Hank's balanced salt solution (HBSS), antibiotics, L-glutamine, sodium pyruvate and trypsin were purchased from Gibco (Invitrogen, Paisley, UK). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). RO 48-8071 was from Enzo Life Sciences AG (Farmingdale, NY, USA). Cholesterol oxidase was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). 5-Carboxyfluorescein diacetate (CFDA), cysteinyl leukotriene C₄ (LTC₄), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), lovastatin, and methyl- β -cyclodextrin (M- β -CD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [³H]LTC₄ was purchased from PerkinElmer (Waltham, MA, USA). Cholesterol was from Avanti Polar Lipids (Alabaster, AL, USA). The rat monoclonal anti-ABCC1 (MRPr1) antibody was obtained from Alexis (Dedham, MD, USA). GF/C short drop filterplates were from Whatman (Kent, UK). OptiPrep was from Axis-Shield PoC AS (Dundee, Scotland).

Cell culture and incubation conditions

The murine neuroblastoma cell line Neuro-2a was purchased from the ATCC (Manassas, VA, USA). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate, under standard incubator conditions (humidified atmosphere, 5% CO₂, 37°C). The hamster BHK cell line stably expressing the human *ABCC1* gene, named BHK-MRP1, was a gift from Dr. Riordan (Mayo Clinic Arizona, S.C. Johnson Medical Research Center, Scottsdale, AZ, USA; ref. 10). These cells were grown as adherent monolayer cultures in Dulbecco's modified Eagle medium/NUT mix F-12 (1:1) supplemented with 10% FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine, under standard incubator conditions (humidified atmosphere, 5% CO₂, 37°C). The cells were kept under selective pressure by growing them in the presence of 100 μ M methotrexate. In order to deplete cholesterol, cells were incubated in the presence of 10 mM M- β -CD for various time intervals in serum-free medium. Alternatively, Neuro-2a cells were incubated for 20 h in the presence of both 1 μ M RO 48-8071, an inhibitor of oxidosqualene cyclase, and 1 μ g/ml lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, in serum-free medium. As a control in this case, cells were grown in serum-free medium for 20 h with vehicle. In order to chemically convert cholesterol to cholestenon, cells were incubated in the presence of 1 U/ml cholesterol oxidase for 1 h. For cell membrane preparations cells were grown in roller bottles, harvested by trypsinization, and then subjected to cholesterol oxidase or M- β -CD treatment for various time intervals to deplete plasma membrane cholesterol. For cholesterol loading of the plasma membrane, M- β -CD filled with cholesterol (M- β -CD/cholesterol) was used for different incubation times in serum-free medium at 37 °C (10 mM concentration). To prepare M- β -CD/cholesterol, 100 mg M- β -CD was dissolved in 2 ml water and 3 mg cholesterol (solution in ethanol) was added slowly while stirring at 60-70 °C. This was dried and used later as described above. After treatment cells were centrifuged (3000 rpm, 3 min), frozen in liquid nitrogen and stored at -80 °C until membrane isolation. Control cells were treated similarly but in the absence of cholesterol modulators, i.e. solvent controlled when applicable.

Isolation of detergent-free lipid rafts

Detergent-free lipid rafts were isolated as described [13,14].

Immunoblot analysis

Protein from equal volumes of the gradient fractions was processed as described [14].

Cholesterol determination

To quantify free cholesterol, a lipid extraction [15] on cells or pooled detergent-free lipid fractions was performed after a protein determination [16]. The cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase/peroxidase assay [17]. The amount of cholesterol was expressed relative to the protein content.

Sphingolipid analysis by liquid chromatography-electrospray ionization tandem mass spectrometry

Sphingolipids were extracted and analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) as described previously [14]. The amounts of individual sphingolipid species were added to obtain the total sphingolipid pool. Protein content of pooled OptiPrep gradient fractions was determined as described by Smith *et al.* [16].

Detection of Abcc1/ABCC1-mediated efflux by flow cytometric analysis

Neuro-2a or BHK-MRP1 cells were plated to confluence in 25 cm² flasks one day prior to the experiment. Abcc1/ABCC1-mediated efflux of the substrate 5-carboxyfluorescein diacetate was performed by flow cytometric analysis as described [18].

Measurement of cellular sensitivity to cytotoxic drugs (MTT assay)

One thousand cells/well were plated in microtiter plates. For depletion of cholesterol, cells were washed 24 h after plating with serum-free medium and incubated in the presence of 10 mM M- β -CD in serum-free medium for 1h at 37°C. Subsequently, cells were washed with serum-free medium and incubated for 48h in serum-containing medium. Viable cells were determined 72h after plating as previously described [14].

Isolation of membrane vesicles from BHK-MRP1 cells

BHK-MRP1 cells were washed with HBSS, trypsinized, harvested and treated, then centrifuged (3000 rpm, 3 min), frozen in liquid nitrogen and stored in -80 °C. Pellets of ~ 8x10⁸ frozen cells were resuspended in hypotonic solution (5 mM sodium phosphate, pH 7.4, containing protease inhibitors and 1 mM EDTA) by stirring for 90 min on ice. After centrifugation (100000 x g, 45 min, 4 °C) cell material was resuspended in isotonic buffer (10 mM Tris, pH 7.4, 250 mM sucrose, containing protease inhibitors and 1 mM EDTA) and homogenized using a Dounce tissue grinder. The material was carefully layered on top of a 38% sucrose solution and centrifuged (280000 x g, 2 h, 4 °C). The membrane interface was collected and diluted in isotonic buffer and centrifuged again (100000 x g, 45 min, 4 °C). The membrane pellet was finally resuspended in isotonic buffer at a concentration of 5 mg/ml of protein [16], frozen in liquid nitrogen and stored at -80 °C.

Measurement of ABCC1-mediated ATPase and transport activities

Vanadate sensitive ATPase activities were measured as described earlier [19,20]. Briefly, isolated membranes (8 μ g/well) were incubated for 11 min in 50 μ l ATPase assay mix (40 mM MOPS-Tris pH 7.0, containing 10 mM MgCl₂, 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EGTA, 4 mM Na-azide, and 5 mM ATP). The reaction was stopped with 100 μ l malachite green mix [21-25], as modified in our lab (5.6 mM malachite green, 9.4 mM ammonium-molybdate, 2 % citric acid, 4.6 % ethanol, and 1.16 N HCl, final concentration). After two

min 100 μ l of citric acid was added and incubated at 37 °C for 15-20 min. The absorbance values were measured using a spectrophotometer (Biotek uQuant) at a wavelength of 630 nm. For transport of cysteinyl leukotriene C₄ (LTC₄) into membrane vesicles, vesicles were incubated as described before [26] in 40 mM MOPS-Tris pH 7.0, 180 mM sucrose, containing 10 mM MgCl₂, 20 mM KCl, 47.8 nM LTC₄ and with or without 4 mM ATP, for various time intervals at 37 °C. LTC₄ at a concentration of 47 nM was mixed with ³H-LTC₄ (specific activity of 190 Ci/mmol) at a concentration of 0.8 nM. The reaction was stopped with ice cold washing buffer (10 mM Tris pH 7.0, 180 mM sucrose, 20 mM KCl). The reaction mix was rapidly filtered in glass fibre filter plates (Whatman GF/C short drop), washed with 5x200 μ l ice cold washing buffer, and dried. Radioactivity was measured by liquid scintillation counting (Packard Topcount microplate scintillation counter). ATPase activity was expressed as pmol Pi/mg/min and transport into membrane vesicles as pmol LTC₄/mg/min. For graphic representation, values were normalized to maximal activity in control conditions (=100%).

Measurement inside-out vesicle content

Determination of the inside-out vesicle ratio was based on 5'-nucleotidase activity and performed as described earlier [27] with the following changes: 25-50 μ g of membrane vesicles was incubated (30 min, 37 °C) in 50 mM Tris, pH 7.4, containing 4 mM MgCl₂, and with or without 3 mM AMP and/or 0.3 % Triton X-100. The inorganic phosphate released by the 5'-nucleotidase was measured with the malachite green system as described above. 5'-nucleotidase activity was measured in 4 conditions; A: with AMP and Triton X-100, B: with AMP, without Triton X-100, C: without AMP, with Triton X-100 and D: without AMP, without Triton X-100. A-C yields the total activity of the enzyme, B-D the activity of the right side-out vesicles only. The percentage of inside out vesicles was calculated as [(A-C)-(B-D)]x100/(A-C).

Results

Efficient depletion or uploading of cholesterol

First we established the efficacy of cholesterol modulation, using three different strategies. 1) M- β -CD was used to physically deplete cholesterol from cell membranes. 2) Lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, plus RO 48-8071, an inhibitor of oxidosqualene cyclase, were used to inhibit the biosynthesis of cholesterol. 3) Cholesterol oxidase was used to chemically convert cholesterol to cholestenon.

The results are summarized in Table 1. In Neuro-2a cells a 1 h treatment with 10 mM M- β -CD decreased the cholesterol level by 71.6% in whole cells. In detergent free lipid raft fractions from these cells, cholesterol was even more strongly depleted (87.0%). In BHK-MRP1 cells cholesterol depletion by M- β -CD was 64.1%. The M- β -CD treatment slightly affected Neuro-2a cell viability, as determined by a MTT assay. Neuro-2a cell viability was $81.4\% \pm 18.6\%$ (n=6) for M- β -CD-treated cells (1 h) compared to control cells (=100%).

Conditions for inhibition of cholesterol biosynthesis in Neuro-2a cells were optimized in terms of concentrations and time of incubation (data not shown). Cells incubated for 20 h in the presence of both 1 μ g/ml lovastatin and 1 μ M RO 48-8071 showed a 52.9% decrease of the cholesterol level. Finally, when 1 U/ml cholesterol oxidase was used for 1 h in Neuro-2a cells, the cholesterol content was reduced by 29.0%. In BHK-MRP1 cells, the enzyme was much more efficient, reducing cholesterol by 96.3%.

The cholesterol levels in cell membranes isolated from cholesterol-modulated BHK-MRP1 cells are summarized in Table 2. Cholesterol oxidase treatment of intact BHK-MRP1 cells resulted in a maximal decrease in cholesterol level of ~50% in the isolated cell membranes. This was less efficient compared to previous experiments (Table 1), possibly due to the use of large scale cell cultures needed for the subsequent isolation of membranes. We also treated isolated membrane vesicles with cholesterol oxidase, but this resulted in degradation of ABCC1 (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/>) and these preparations were therefore not suitable for analysis of ABCC1 function. ABCC1 was not degraded in membrane preparations obtained from cholesterol-modulated BHK-MRP1 cells, as compared to control membrane preparations (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/>).

Cholesterol depletion by M- β -CD treatment reduced cholesterol by ~57% in isolated membranes, while cholesterol uploading with M- β -CD/cholesterol increased the cholesterol level by ~58%. The cholesterol content of control membrane vesicles was 240 ± 30 nmol cholesterol/mg protein (n=9).

Cholesterol does not affect efflux function of Abcc1/ABCC1

Having established the extent of cholesterol modulation with three different strategies, we next measured the effects on Abcc1/ABCC1-mediated CFDA efflux. Efflux activity was the same in cholesterol oxidase-treated and control cells, regarding both Neuro-2a (Fig. 1A) and BHK-MRP1 (Fig. 1B) cells. Also in lovastatin/RO 48-8071-treated Neuro-2a cells, efflux activity was similar to control (Fig. 1C). MK571 was used as a positive control for inhibition of Abcc1/ABCC1-mediated efflux (Fig. 1A-C). With M- β -CD differential effects on Abcc1/ABCC1-mediated efflux kinetics were observed in the two cell types. In Neuro-2a cells, efflux activity was normal up to 10 min of M- β -CD treatment, but was reduced after 30 min and maximally affected after 45 min treatment (Fig. 2A). In BHK-MRP1 cells, on the other hand, ABCC1-mediated efflux activity was already reduced after 10 min of M- β -CD treatment, and subsequently kept fluctuating around this level (Fig. 2B). It was therefore important to measure in parallel the kinetics of cholesterol depletion in both cell types, which turned out to be very similar in Neuro-2a and BHK-MRP1 cells (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/>). Cholesterol levels were equally decreased at t = 10 min in both

cell types (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/>). Interestingly, upon longer M- β -CD treatment, both cell types became sensitive to MK571, as indicated by loss of Abcc1/ABCC1 substrate likely due to membrane leakage. This occurred between 45 and 60 min for Neuro-2a (Fig. 2C) and between 15 and 30 min for BHK-MRP1 cells (Fig. 2D).

Abcc1/ABCC1 efflux function is not correlated with its localization in lipid rafts

We used a detergent-free method for the isolation of lipid rafts and first characterized the gradient fractions in terms of cholesterol and sphingolipid enrichment. For this purpose, fractions 1-2 were pooled, as well as 3-4, 5-6 and 7-9. Fractions 1-2 were most strongly enriched in both cholesterol and sphingolipids (Supplementary Figure S3 at <http://www.BiochemJ.org/bj/>), and to a lesser extent also fractions 3-4. This indicates that fractions 1-2, with the lowest buoyant density, optimally fulfil the criteria for lipid rafts. In accordance with the absence of an effect of cholesterol oxidation on Abcc1 efflux function, there was no effect on detergent-free lipid raft localization of the ABC transporter, as indicated by a similar gradient profile of Abcc1 compared to control Neuro-2a cells (Fig. 3A). Cholesterol oxidase was much more effective in reducing cholesterol content in BHK-MRP1 cells than in Neuro-2a cells. Therefore, we also rigorously tested lipid raft association of ABCC1 under cholesterol oxidase conditions in BHK-MRP1 cells and this turned out to be equal to control cells, as confirmed by quantification of the lipid raft-associated pools (Fig. 3B).

On the other hand, lovastatin/RO 48-8071 treatment did result in a clear shift of Abcc1 out of detergent-free lipid raft fractions in Neuro-2a cells, as confirmed by quantification of the relative amount of Abcc1 in lipid raft fractions (Fig. 3C). Upon M- β -CD treatment, detergent-free lipid raft association of Abcc1 was gradually reduced in Neuro-2a cells and this was significant after 60 min (Fig. 4A). In BHK-MRP1 cells, ABCC1 showed a tendency to shift out of lipid raft gradient fractions only after 60 min M- β -CD treatment, but this did not reach significance due to large variation (Fig. 4B).

ABCC1 ATPase and transport activities are not correlated with cholesterol levels in isolated cell membranes

The absence of effects of cholesterol modulation on Abcc1/ABCC1 function could have been due to confounding factors in the complex background of the intact cell. To overcome the complexity of the cell system, plasma membrane vesicles were isolated from cholesterol modulated BHK-MRP1 cells. To make sure that our procedures to modulate cholesterol in cells did not affect the inside-out ratio of ABCC1 in the isolated membrane vesicles, we measured this ratio. Under all conditions, the inside-out vesicle ratio was found to be invariable (Supplementary Figure S4 at <http://www.BiochemJ.org/bj/>). Moreover, ABCC1 integrity was not changed by our cholesterol modulation procedures, as shown by Western blot (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/>).

In this simplified system for ABCC1 function analysis, we measured ATPase activity of ABCC1 as well as transport of LTC₄ into membrane vesicles by ABCC1 (Fig. 5). The average vanadate sensitive ATPase activity and transport of LTC₄ into isolated control plasma membranes were 34 \pm 9 pmol Pi/mg/min (n=27) and 74 \pm 39 pmol/mg/min (n=27), respectively. There were no significant differences between cholesterol depleted (cholesterol oxidase or M- β -CD treated) or cholesterol uploaded (M- β -CD/cholesterol treated) membranes on the one hand and control membranes on the other (Fig. 5 and Supplementary Table S1 at <http://www.BiochemJ.org/bj/>). The average V_{max} and K_m values of the transport of LTC₄ into membrane vesicles were 661 \pm 171 pmol/mg/min and 942 \pm 220 nM, respectively. These kinetic parameters of the LTC₄ transport did not show any statistical differences between cholesterol-modulated and control membranes (data not shown).

Discussion

With regard to cholesterol-modulated ABC transporter activity, most studies have focused on P-glycoprotein. These studies have generated much information, but unfortunately not a coherent picture of whether and how cholesterol affects P-glycoprotein function [6,28]. Concerning modulation of Abcc1/ABCC1 by cholesterol, very little information is available. One study shows that when the cellular cholesterol level is lowered, ABCC1 function is reduced and concomitantly ABCC1 shifts out of lipid rafts in GLC4 multidrug-resistant lung cancer cells [9]. This conclusion was based on results obtained solely with M- β -CD treatment. In our study, we obtained similar effects of M- β -CD treatment. However, we did not attribute these effects to cholesterol, in view of the results obtained with the additional procedures for cholesterol modulation, i.e. cholesterol oxidase and lovastatin plus RO 48-8071.

Abcc1/ABCC1-mediated efflux was not affected in these two treatment protocols, while the cholesterol level was strongly decreased, especially in cholesterol oxidase-treated BHK-MRP1 cells. Moreover, with M- β -CD treatment efflux was dissociated from the cholesterol level, since short-term treatment did affect efflux in BHK-MRP1, but not in Neuro-2a cells, while cholesterol levels were equally decreased in the two cell types. Long-term M- β -CD treatment in Neuro-2a cells decreased both cholesterol levels and Abcc1-mediated efflux. However, long-term M- β -CD treatment has been shown to result in side effects, such as removal of other lipids and potentially even proteins from membranes [8,29]. Thus, interpretation of such studies should be done with care. The data obtained with MK571 as inhibitor of Abcc1/ABCC1 efflux show that after short-term M- β -CD treatment the residual cell associated substrate is high, as expected. However, after long-term M- β -CD treatment substrate is lost from MK571-treated cells, suggesting that MK571 becomes toxic in combination with M- β -CD. This would indicate that membranes gradually destabilize with M- β -CD treatment. It is noteworthy that the M- β -CD effect becomes apparent at shorter incubation times in BHK-MRP1 cells, as compared to Neuro-2a cells. This could be related to the fact that BHK-MRP1 cells were subjected to forced expression of human ABCC1, possibly resulting in less stable integration of ABCC1 in the plasma membrane. As a consequence, ABCC1 would become more prone to the destabilizing effects of M- β -CD. On the other hand, when the M- β -CD effect on Neuro-2a cells sets in, it is more pronounced compared to that in BHK-MRP1 cells.

The studies on intact cells were extended to isolated membrane vesicles to overcome potential confounding effects in the complex system of the intact cell. The modulation of cholesterol performed on the cells did not change the integrity and inside-out ratio of ABCC1 in the vesicles consisting of isolated membranes from these cells, allowing us to properly measure potential effects on ABCC1 function. Data on ABCC1 function in these vesicles with decreased cholesterol levels confirm the conclusion that ABCC1 function does not depend on cholesterol. In addition, uploading of cholesterol was ineffective with regard to the function of ABCC1. Moreover, this shows that our results are not specific for the Abcc1/ABCC1 substrate CFDA, since in this assay for ABCC1 function in vesicles from isolated membranes a completely different substrate (LTC₄) was used.

Next we explored whether Abcc1/ABCC1-mediated efflux activity was related to localization of Abcc1/ABCC1 in cholesterol-stabilized lipid rafts. Comparison of results revealed that all 4 combinations of “effect/no effect” on these two parameters occurred. 1) After cholesterol oxidase treatment, both Abcc1/ABCC1-mediated efflux and its localization in lipid rafts were unaffected. 2) Upon long-term M- β -CD treatment both efflux and lipid raft localization of Abcc1/ABCC1 were reduced. 3) On the other hand, with short-term M- β -CD treatment in BHK-MRP1 cells, efflux was affected, while raft localization was normal. 4) The opposite occurred after lovastatin plus RO 48-8071-treatment, which resulted in a shift of Abcc1 out of lipid raft fractions without any effect on efflux activity. Given the fact that all

possible combinations of “effect/no effect” on these 2 parameters occurred, we can conclude that Abcc1/ABCC1-mediated efflux activity and its localization in cholesterol-stabilized lipid rafts were not correlated. In a recent study we showed that Abcc1/ABCC1 efflux function was correlated with its localization in cortical actin-stabilized lipid rafts [18]. Therefore, we should not only consider that Abcc1/ABCC1 (and other ABC transporters as well) may behave differently between various cell types in terms of (lipid raft) localization-function coupling. In addition, it may well be that within a certain cell type, different types of lipid rafts exist, which are sensitive to either cholesterol or cortical actin, while only the latter is relevant for ABCC1 function.

Taken together, we thoroughly investigated potential effects of cholesterol on Abcc1/ABCC1 function in two different cell lines and in vesicles from isolated membranes. Moreover, in order not to rely completely on M- β -CD treatment we used 3 different cholesterol modulation procedures. Based in this comprehensive approach, we show for the first time that Abcc1/ABCC1 function can not be categorically linked to cholesterol levels or cholesterol-stabilized lipid raft localization in two different cell lines. A previous study did show an effect on ABCC1 function and localization by lowering the level of cholesterol in yet another cell line [9]. Therefore, studies in additional Abcc1/ABCC1 (over)expressing cell types have to be performed before more general conclusion can be drawn. In any case, cholesterol is not essential for ABCC1 function and therefore does not seem to take part in the mechanism underlying functional association of ABCC1 with lipid rafts, as has been observed for cortical actin [18].

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Figure legends

Figure 1. Cholesterol modulation does not affect Abcc1/ABCC1-mediated efflux in Neuro-2a and BHK-MRP1 cells

A,B: Neuro-2a (A) or BHK-MRP1 cells (B) were untreated (control) or treated with 1 U/ml cholesterol oxidase for 1 h (CO). C: Neuro-2a cells were untreated (control: 20 h in serum-free medium) or treated with 1 µg/ml lovastatin + 1 µM RO 48-8071 for 20 h in serum-free medium (LO/RO). Subsequently, cells were loaded with CFDA (0.5 µM) and allowed to efflux at 37°C during several time intervals. The remaining cell-associated fluorescence was determined by cytometric analysis and expressed as % of the 0 min value. Control: open squares; CO or LO/RO: closed squares. MK571 (20 µM) was used as a positive control for Abcc1/ABCC1 efflux inhibition. Control + MK571: open triangles; CO + MK571 or LO/RO + MK571: closed triangles. Data represent the mean ± SD (n=3).

Figure 2. Effects of M- β -CD treatment on Abcc1/ABCC1-mediated efflux in Neuro-2a and BHK-MRP1 cells

Neuro-2a (A,C) or BHK-MRP1 cells (B,D) were treated with 10 mM M- β -CD for various time intervals. Subsequently, cells were loaded with CFDA (0.5 μ M) and allowed to efflux at 37°C for 5 min. The remaining cell-associated fluorescence was determined by cytometric analysis and expressed as % of the 0 min value. MK571 (20 μ M) was used as a control for Abcc1/ABCC1 efflux inhibition (C,D). Data represent the mean + SD (n=3).

Figure 3. Detergent-free lipid raft localization of Abcc1 is affected by lovastatin/RO 48-8071 but not by cholesterol oxidase in Neuro-2a cells (and BHK-MRP1 cells)

Cells were untreated (CON) or treated with either 1 U/ml cholesterol oxidase for 1 h (CO) or with 1 µg/ml lovastatin + 1 µM RO 48-8071 for 20 h in serum-free medium (LO/RO). A: Lipid raft association of Abcc1 in CO-treated Neuro-2a cells compared to control. B: Lipid raft association of ABCC1 in CO-treated BHK-MRP1 cells compared to control. C: Lipid raft association of Abcc1 in LO/RO-treated Neuro-2a cells compared to control. The numbers indicate the percentage of Abcc1/ABCC1 found in the pooled gradient fractions, relative to total Abcc1/ABCC1 in the entire gradient (9 fractions). Data represent the mean + SD (n=3). Asterisks indicate values that are significantly different from CON (P < 0.05, as determined by Student's *t*-test). The right panels show representative blots from three experiments concerning Abcc1/ABCC1 distribution along the gradient. Gradient samples were applied on SDS-PAGE based on equal volume. Lipid raft fractions are indicated.

Figure 4. Effects of M- β -CD treatment on detergent-free lipid raft localization of Abcc1/ABCC1 in Neuro-2a and BHK-MRP1 cells

Neuro-2a (A) or BHK-MRP1 cells (B) were untreated (0 min) or treated with 10 mM M- β -CD for various time intervals (5, 30, 60 min) and lipid raft association of Abcc1/ABCC1 was determined under these conditions. The numbers indicate the percentage of Abcc1/ABCC1 found in the gradient fractions, relative to total Abcc1/ABCC1 in the entire gradient (9 fractions). Data represent the mean + SD (n=3). Asterisks indicate values that are significantly different from CON ($P < 0.05$, as determined by Student's *t*-test). The right panels show representative blots from three experiments concerning Abcc1/ABCC1 distribution along the gradient. Gradient samples were applied on SDS-PAGE based on equal volume. Lipid raft fractions are indicated.

Figure 5. Vanadate sensitive ATPase activity and transport of LTC₄ into membrane vesicles by ABCC1 in cholesterol modified membrane vesicles

BHK-MRP1 cells were treated with cholesterol oxidase (CO; 1 U/ml; A,B), M- β -CD (CD; 10 mM; C,D), or M- β -CD/cholesterol (CDC; 10 mM; E,F). Cell membranes were isolated from these cells and control cells. The vanadate sensitive ATPase activity (A,C,E) or the transport of LTC₄ into membrane vesicles (B,D,F) by ABCC1 were measured. For each treatment, three independent membrane preparations were generated. For each membrane preparation, the measurements were performed in triplicate. Each data point in a curve represents the average \pm SD of three independent membrane preparations. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min. (a) \blacklozenge solid line; (b) \blacksquare dotted line; (c) \blacktriangle dashed line; (d) \bullet dashed dotted line. ATPase activity was expressed as pmol Pi/mg/min and LTC₄ transport as pmol LTC₄/mg/min. For graphic representation, values were normalized to maximal activity in control conditions (=100%).

TABLES

Cell type	Fraction	Method	Cholesterol content (control = 100%)
Neuro-2a	whole cells	M- β -CD	28.4% \pm 12.3
Neuro-2a	detergent-free lipid rafts	M- β -CD	13.0% \pm 7.5
Neuro-2a	whole cells	lovastatin + RO 48-8071	47.1% \pm 7.8
Neuro-2a	whole cells	cholesterol oxidase	71.0% \pm 5.0
BHK-MRP1	whole cells	M- β -CD	35.9% \pm 3.0
BHK-MRP1	whole cells	cholesterol oxidase	3.7% \pm 2.1

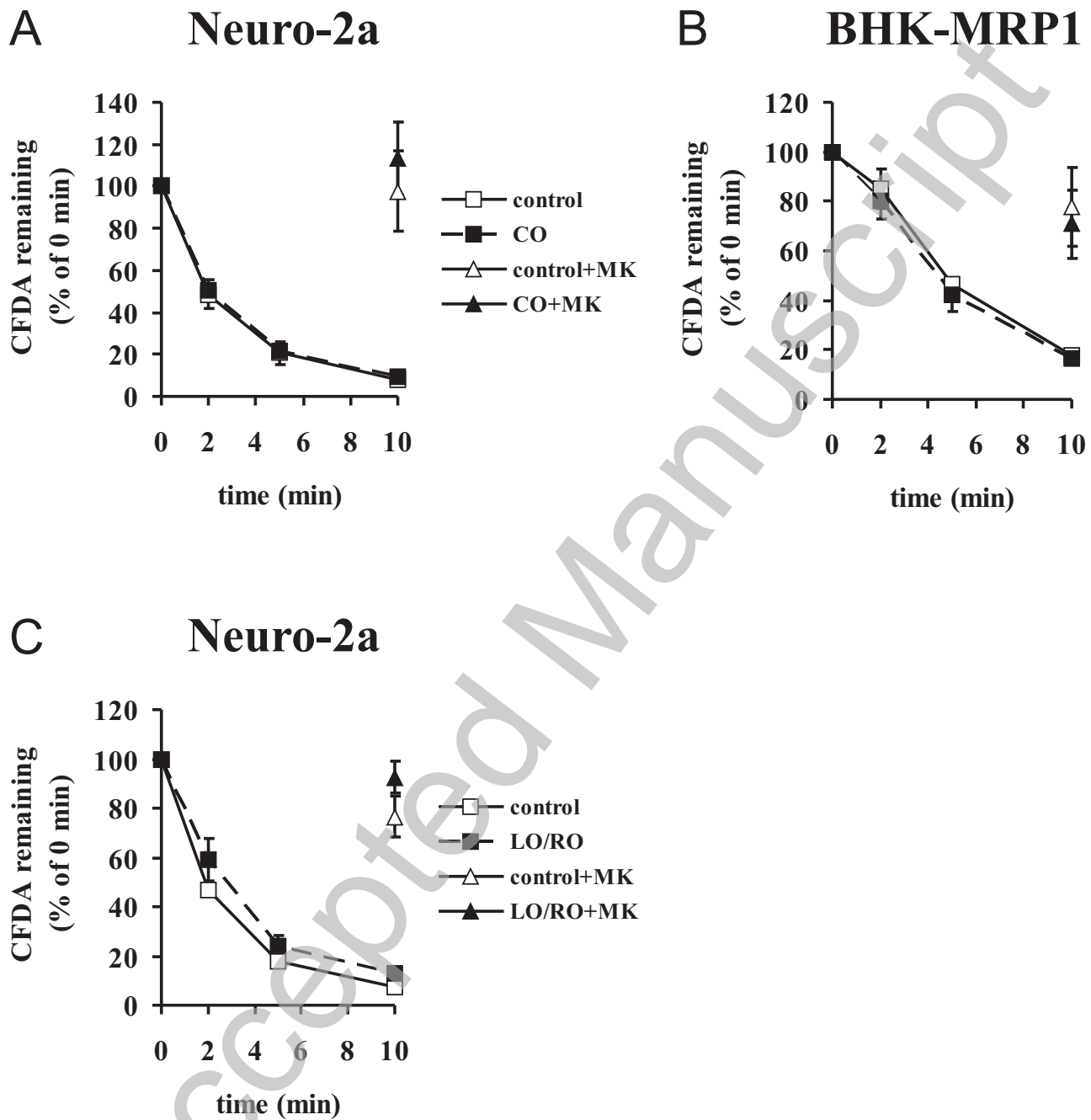
Table 1: Cholesterol depletion methods and efficiency in intact cells

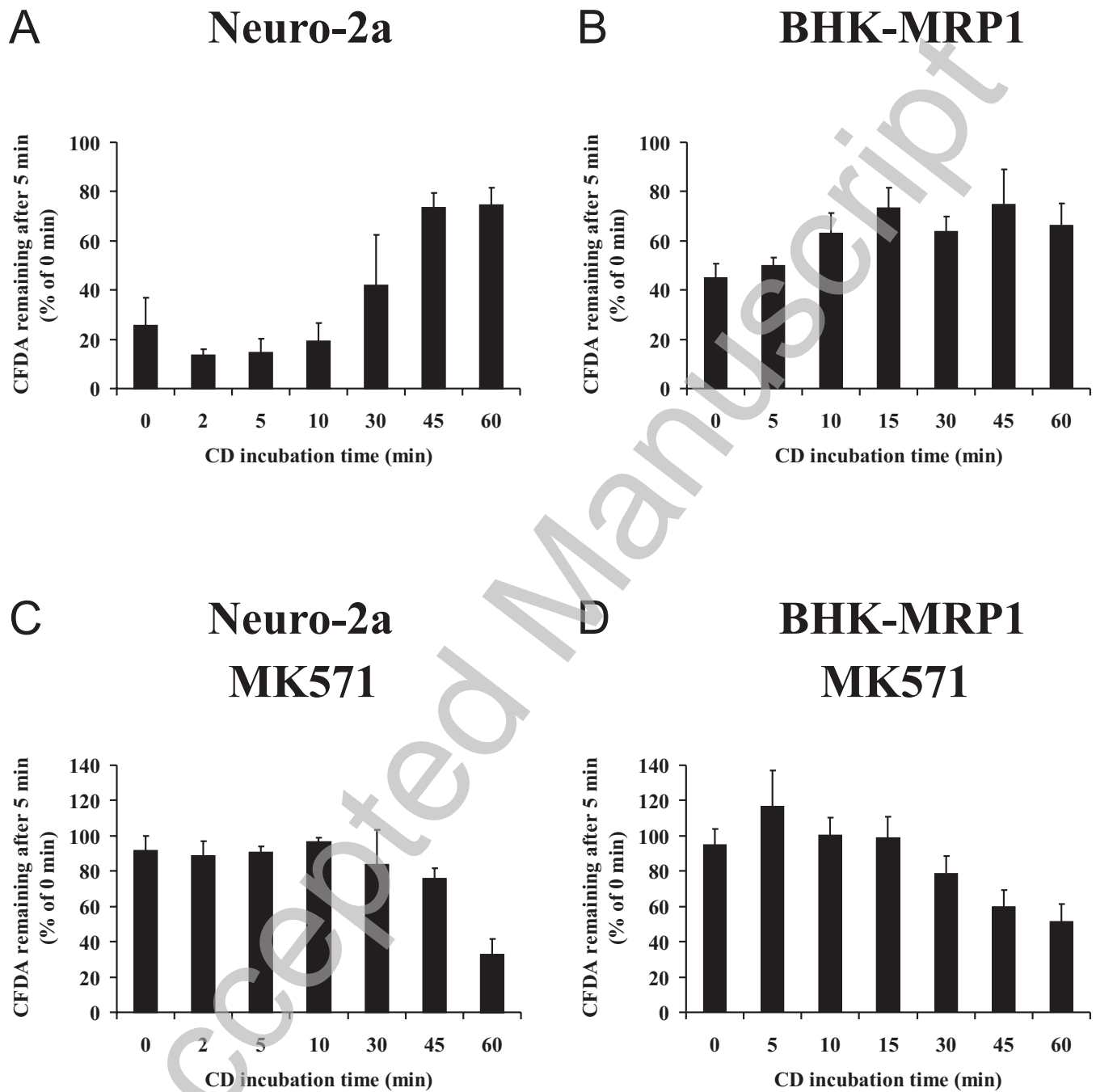
Various procedures were performed to manipulate cholesterol levels, including M- β -CD treatment (10 mM, 1 h), which physically removes cholesterol from the plasma membranes of cells, cholesterol oxidase treatment (1 U/ml, 1 h), which chemically converts cholesterol into cholestenon and combined treatment with lovastatin (1 μ g/ml), an inhibitor of 3-hydroxy-3methylglutaryl-coenzyme A reductase and RO 48-8071 (1 μ M), an inhibitor of oxidosqualene cyclase. Cholesterol content was measured and normalized to protein. These values were normalized to control (=100%). Data indicate the mean \pm SD of three independent measurements.

Cholesterol depletion with Cholesterol Oxidase (CO)		Cholesterol depletion with M- β -Cyclodextrin (CD)		Cholesterol upload with M- β -CD/cholesterol (CDC)	
Treatment	Cholesterol (%)	Treatment	Cholesterol (%)	Treatment	Cholesterol (%)
COa	100	CDa	100	CDCa	100
COb	75 \pm 1,8	CDb	71 \pm 4,8	CDCb	136 \pm 15,2
COc	63 \pm 6,8	CDc	53 \pm 2,6	CDCc	143 \pm 17,7
COd	52 \pm 1,2	CDd	43 \pm 5.1	CDCd	158 \pm 23,3

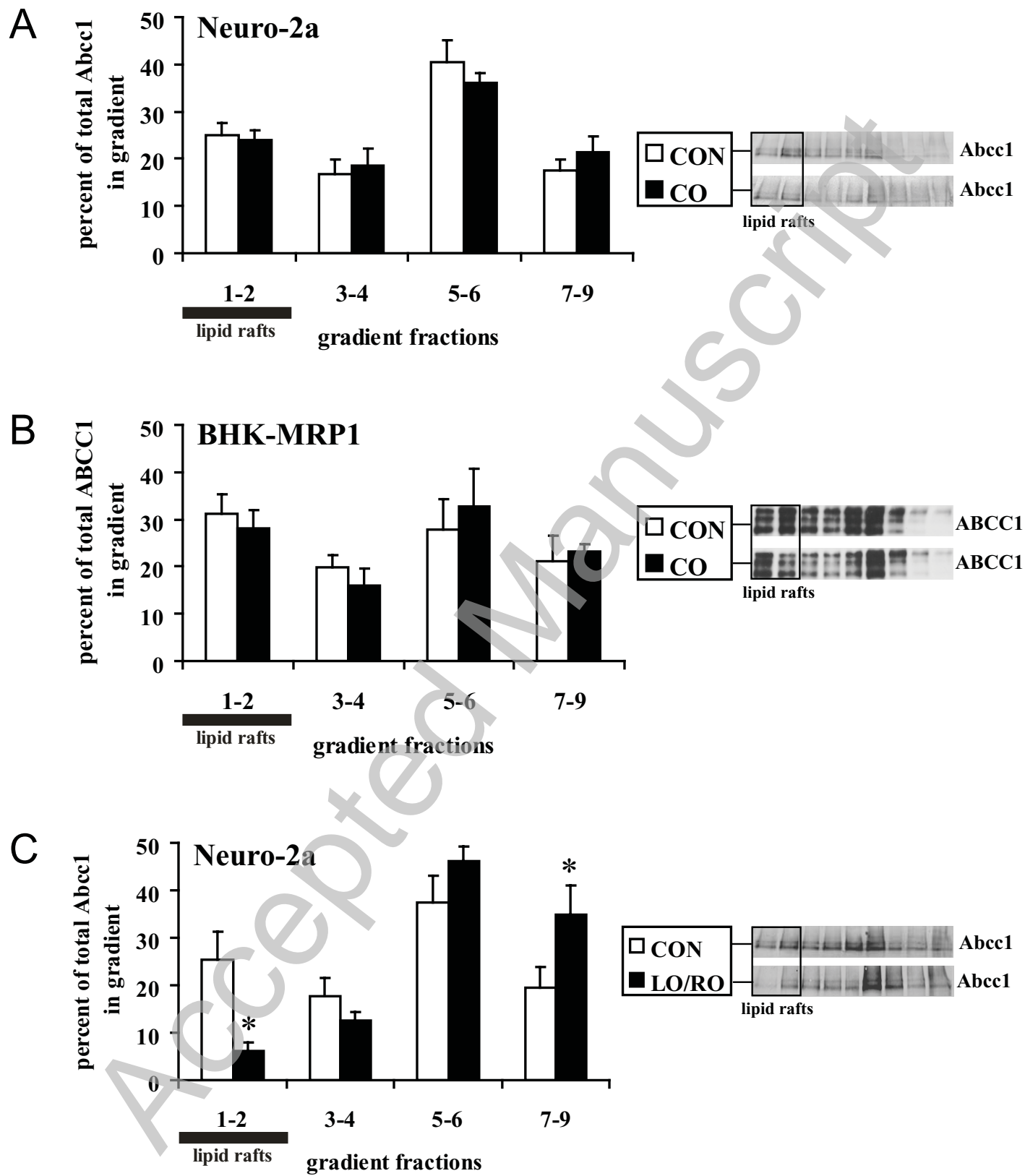
Table 2: Cholesterol modulation methods and efficiency in isolated membranes from BHK-MRP1 cells

BHK-MRP1 cells were treated during various time intervals with cholesterol oxidase (CO; 1 U/ml), M- β -CD (CD; 10 mM), or M- β -CD/cholesterol (CDC; 10 mM). Each treatment was performed independently three times, resulting in three independent membrane preparations. Cholesterol content was measured and normalized to protein. These values were normalized to control (=100%). Data indicate the mean \pm SD of three independent membrane preparations. (a) represents membranes from control cells and (b), (c) and (d) membranes from cells treated for various time intervals with CO, CD or CDC, respectively. In case of CO, (b), (c) and (d) refer to 30, 60 and 90 min treatment, respectively. In case of CD and CDC, this is 10, 20 and 30 min.

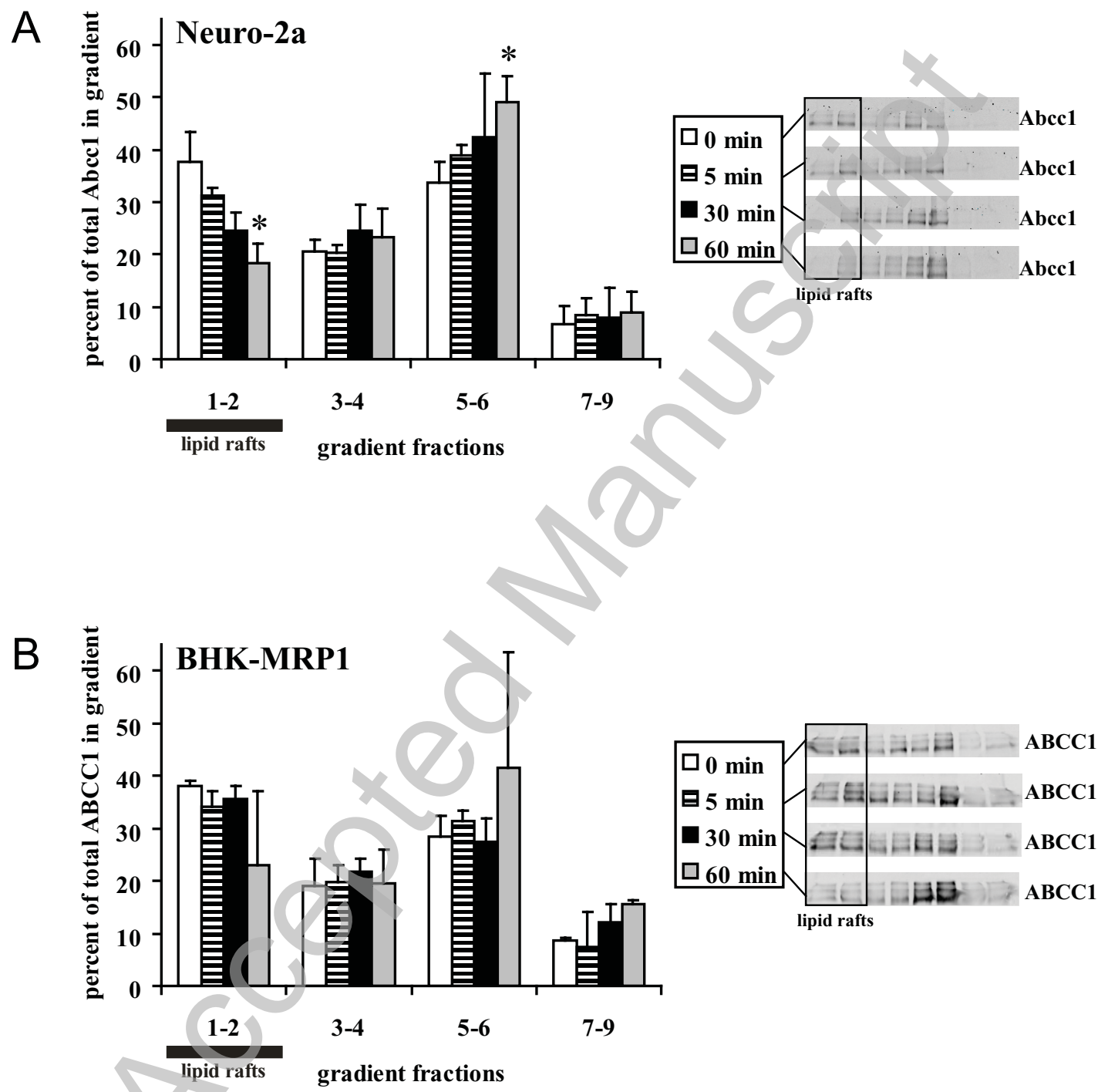
**Figure 1**

**Figure 2**

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**Figure 3**

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**Figure 4**

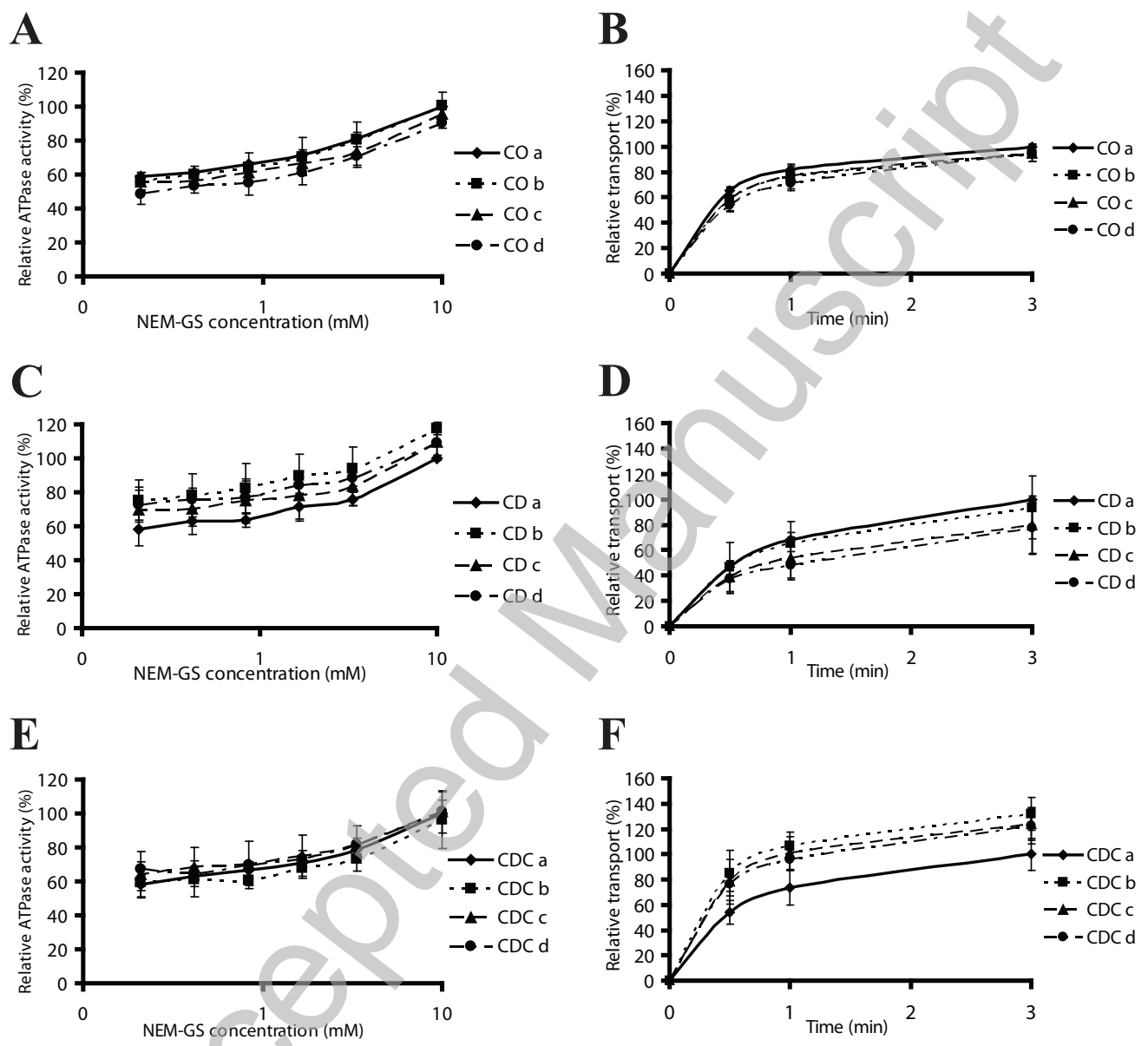


Figure 5

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