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Characterization of the ATPase Activity of Human ATP-binding Cassette Transporter-2 (ABCA2)

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Abstract

Background: ABCA2 is a member of the ATP binding cassette transporter family with functional roles in cholesterol homeostasis and drug resistance.

Materials and Methods: In order to characterize its ATPase activity, we transfected HEK293 cells with an ABCA2 mammalian expression system and isolated ABCA2-enriched membranes.

Results: We found no measurable ATPase activity of ABCA2 in isolated membranes, except in the presence of the methyl-β-cyclodextrin. However, competitive binding of a pseudo-substrate, 8-azido-[α-^{32}P]-ATP, was demonstrated. CHO cells transfected with ABCA2 did not have a higher rate of endogenous ATP hydrolysis when compared to the mock-transfected cells.

Conclusion: Overall, we conclude that, while ABCA2 may have low levels of ATPase activity that can be substrate-stimulated, it is more likely to have a regulatory role in cell physiology.

Keywords

ABCA2 transporter; ATPase activity; pseudo-substrate; catalysis

ABC transporters comprise one of the largest gene families, encoding more than 50 known structurally related trans-membrane transporters. Evolutionarily conserved between bacteria and humans, these transporters have broad substrate specificities and participate in various physiological functions in humans, from transport to signaling (19). Transport substrates can include amino acids, nucleotides, steroids, peptides, toxins, xenobiotics and their conjugates (5, 6, 13). The majority of the ABC transporters in humans are made of one polypeptide chain, with two distinct types of domains, trans-membrane and nucleotide binding (1, 13).

According to their physiological function, ABC transporters can be roughly classified into two distinct categories: energy-dependent, unidirectional transport of small molecules (*e.g.* MDR1, MRP1–5) and channels with regulatory functions (*e.g.* CFTR, SUR1–2). However,

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this categorization is not stringent, and it is plausible that ABC transporters may have bifunctional roles. Binding and hydrolysis of ATP by some transporters yields energy for the transport of small molecules across membranes (14). The exact mechanism(s) that couple ATP hydrolysis and molecule movement is currently unknown. Therefore, much of the research has been focused on the characterization of the ATPase cycle of different transporters.

ABCA2 expression has been linked to estramustine resistance, through an observed gene amplification on chromosome 9q34 in an estramustine-resistant cell line (18), and with resistance to the anticancer drug mitoxantrone (4). ABCA2 shares 44% sequence homology with ABCA1 and, although primarily localized in different cell compartments, both proteins have been functionally linked with lipid metabolism (10, 11).

To date, no information on the ATPase activity of ABCA2 has been published. Independent of substrate specificity, such data could help in elucidating whether ABCA2 has a regulatory or active transporter role in cell homeostasis. Previous studies have established that its family member, ABCA1, although implicated in lipid transport, does not have measurable ATPase activity (24). However, these results were generated in transfected insect cells that are not competent to carry out mammalian N-glycosylation of membrane transporters (24). Therefore, the present report measured the ATPase activity of ABCA2 in mammalian cell lines, HEK293 or CHO cells.

Materials and Methods

The full-length (7434 base-pair) wild-type ABCA2 cDNA fragment (isoform b) was subcloned into pcDNA5/FRT vector (Invitrogen) with cytomegalovirus promoter (10). Chinese hamster ovary (CHO) cells were stably transfected using FlpIn System (Invitrogen), according to the manufacturer's instructions. Cells were grown in MEM/Ham's F12 media with 10% FBS, 1% Penn-Strep, 2 mM L-glutamine and 750 μg/ml hygromycin for selection (15). Mouse skin fibroblasts (NIH/3T3), stably transfected with the ATP-binding cassette transporter MRP1 were created as described previously (7), and grown in DMEM (GIBCO) supplemented with 10% FBS, 1% Pen-Strep and 2 mM L-glutamine.

Mutations of the conserved lysine to methionine in the Walker A motif of the two nucleotide binding domains (L1031M and L2094M) were described elsewhere (15). The ABCA2–2LM fragment bearing two L to M mutations was subcloned into pcDNA5/FRT vector and hygromycin-resistant colonies were analyzed for ABCA2 expression, as described above.

Polyclonal rabbit antiserum was raised against a synthetic peptide corresponding to residues 1499–1522 of the primary ABCA2 sequence. Protein levels in whole cell lysates were quantified by Bradford assay (Bio-Rad), separated by 6% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% BSA in Tris-buffered saline and 0.1% Tween 20 and probed for 1 h at room temperature with anti-ABCA2 antibody (25). Antirabbit-horseradish peroxide (Amersham Pharmacia Biotech NA934, Uppsala, Sweden) was used as the secondary antibody. Bands were visualized by exposing the nitrocellulose membrane to film (Kodak).

Membrane vesicles were prepared by the nitrogen cavitation method (9): 10^8 cells were cavitated under nitrogen at 400 psi for 15 min, and isolated by differential centrifugation over a sucrose gradient. ATP crosslinking and ATPase assays were performed as described previously (23) with minor modifications. For ATP cross-linking, 10 μg (total protein) of membrane vesicles was incubated with 5 μCi of 8-azidoadenosine -5-[α-^{32}P]-triphosphate (ALT Inc.) at 0°C for 10 min, exposed to UV light for another 10 min and separated by 6 % SDS-PAGE.

The ATPase assay with isolated membranes was performed by incubating vesicles with MgATP in the presence of 5 mM ATP and ATPase assay buffer (50 mM MES-Tris, pH 6.8, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM dithiothreitol and 10 mM $MgCl_2$). The amount of released phosphate was quantified as described previously (22). The amount of intracellular ATP was measured as an indirect parameter of the ATPase activity (11, 20) by the ATP determination kit (Molecular Probes). Cells were harvested at 80–90% confluency and resuspended in PBS solution to a density of 2.5×10^4 cells/ml. Aliquots (0.5 ml) were taken at different time-points, boiled for 5 min, and 10 μl of the cell suspension mixed with 90 μl of the luciferase solution. The amount of intracellular ATP was determined by plotting the values of luminescence against those for ATP standard solutions.

Results and Discussion

Expression of ABCA2 in HEK293 cells.

In order to study ATPase activity, isoform b (15) of the full-length ABCA2 transporter was sub-cloned into the pcDNA3.1 vector and lipid membranes from HEK293 cells were prepared using the nitrogen cavitation and differential centrifugation method. To confirm that stably transfected cells overexpressed full-length ABCA2, cells were lysed and the proteins separated by gel electrophoresis. The expression of a protein band of ~270 kDa (Figure 1), absent in control cells transfected with empty vector, was confirmed as ABCA2 through immunoblot analysis. Using the polyclonal anti-ABCA2 antibody, one band with a mass corresponding to ~270 kDa was visualized (Figure 1). No such band was detected in the empty vector control cells. These results demonstrated that full-length ABCA2 is expressed in HEK293 cells.

Binding of pseudo-substrate to ABCA2.

ABCA1, when expressed in SF9 insect cells and embedded in lipid membranes, binds the pseudo-substrate 8-azido-[α-^{32}P]-ATP in the presence of Mg^{2+} ions (24). To establish if ABCA2 binds to the ATP pseudo-substrate, we isolated lipid membranes from control and cells over-expressing this transporter. After incubation of the membranes with 8-azido-[α-^{32}P]-ATP under non-hydrolytic conditions (0°C) for 10 min and concomitant UV irradiation in the presence of substrate, the membranes were extensively washed, subjected to gel electrophoresis, transferred to PVDF membranes and exposed to film for visualization. In the autoradiogram shown in Figure 2, only in the ABCA2-transfected cells was a band corresponding to the molecular mass of ABCA2 observed, indicating binding of 8-azido-[α-^{32}P]-ATP to ABCA2. We confirmed that the band was ABCA2 by probing the same blot with the anti-ABCA2 antibody (data not shown). ATP, ADP and GTP (but not

AMP, which as an end-product of hydrolysis is not a competitive inhibitor (12)) were shown to act as competitive inhibitors of this binding. These results are consistent with the conclusion that ABCA2 is capable of binding of 8-azido-[α-^{32}P]-ATP and that this binding is specific, at least with respect to competitive nucleotide substrates.

Characterization of the ATPase activity of ABCA2 in isolated membranes.

In analogy with experiments previously reported for ABCA1 (24), we incubated cell membranes for 1 h at 37°C with ATP, but did not observe a statistically significant difference in phosphate concentration upon exposure of cell membranes from either ABCA2-enriched (A16), or control cells (C1) to ATP (Figure 3A). Addition of various ATPase inhibitors, vanadate, sodium azide or ouabain, did not affect the concentration of phosphate ions (data not shown). However, addition of methyl-β-cyclodextrin led to an increase in the concentration of phosphate ions, when either ATP or GTP was used as a substrate (Figure 3B). Methyl-β-cyclodextrin can solubilize cholesterol from cellular membranes (16, 17). Thus, a plausible explanation for the data is that cholesterol might act as a regulator of the ATPase activity of ABCA2. It is known from *in vitro* studies that some primary active transporters (ABCC1 and ABCC2) have very low basal ATPase activity in isolated membranes, but addition of substrates can substantially increase the rate of ATP hydrolysis (2, 3). Therefore, the observed increase in ATPase activity may be a result of the capacity of methyl-β-cyclodextrin to interact with the lipids altering membrane topology. This would predict that either methyl-β-cyclodextrin or a solubilized form of cholesterol is a substrate for ABCA2 when the transporter is embedded in a membrane environment, as is represented by the present results.

To investigate if ABCA2 is capable of catalytic hydrolysis of the occluded form of ATP, we measured phosphate release upon addition of 8-azido-ATP to membrane-embedded ABCA2. Active transporters such as MDR1 and MRP1 have high catalytic turnover and the trapping of an intermediate is possible only in the presence of orthovanadate or aluminum fluoride. Upon incubation of ABCA2 and 8-azido-ATP for 1 h at 37°C, no increase in concentration of phosphate was observed (data not shown), indicating that the endogenous ATPase activity of ABCA2 might be too low for detection by these experimental conditions.

In order to establish if the rate of endogenous phosphate consumption was influenced by ABCA2 (*i.e.* if ABCA2 has measurable ATPase activity *in vivo*), we used cells transfected with ABCA2, a mutant construct ABCA2–2LM or an empty vector. To determine decay rates, intracellular concentrations of ATP were measured at 0, 1, 2, 3 and 4 h. Cells transfected with MRP1 were used as a positive control. As shown in Figure 4, cells transfected with MRP1 had a higher rate of ATP hydrolysis/turnover compared to all other cell lines. No differences in the rate of ATP hydrolysis were observed for the cells transfected with ABCA2, ABCA2–2LM or the empty vector. These data indicate that increased expression of an active ATPase transporter (MRP1) leads to a faster rate of ATP hydrolysis *in vivo*. In addition, increased expression of ABCA2 or a mutant form, ABCA-2LM, did not influence the rate of intracellular ATP consumption.

Overall, our data indicate that, quantitatively, the ATPase activity of ABCA2 is marginal and as such seems to resemble ABCA1. Therefore, ABCA2, similar to ABCA1, may possess

regulatory characteristics *in vivo*. Similar roles have been established for other ABC transporters, including SUR and CFTR (21, 24). Perhaps more importantly, our data suggest that the lack of measurable ATPase activity indicates that the two ATP-binding cassettes might not be attractive domains for drug targeting with small molecule antagonists.

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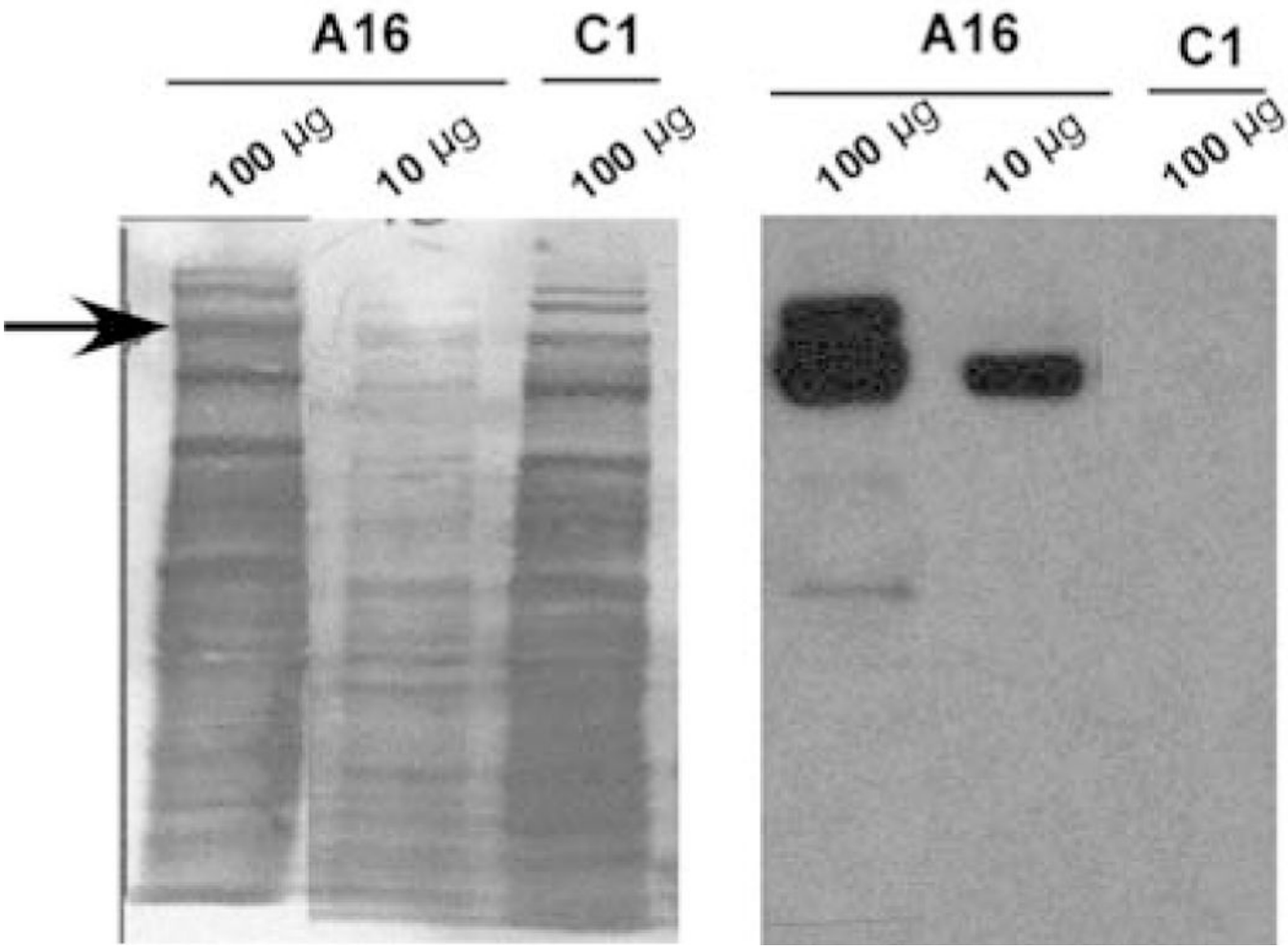

Figure 1.

Expression of ABCA2 (isoform b) in HEK293 cells. Total protein extract (left panel) stained with coommasie blue from HEK cells expressing ABCA2 (A16) or mock-transfected cells (C1). Total amounts of protein loaded are indicated above lanes. Western blot of the membrane using polyclonal anti-ABCA2 antibody as a first antibody (25) and a rabbit IgG conjugated with horseradish peroxidase (right panel). An arrow indicates molecular weight of ~270,00 kDa.

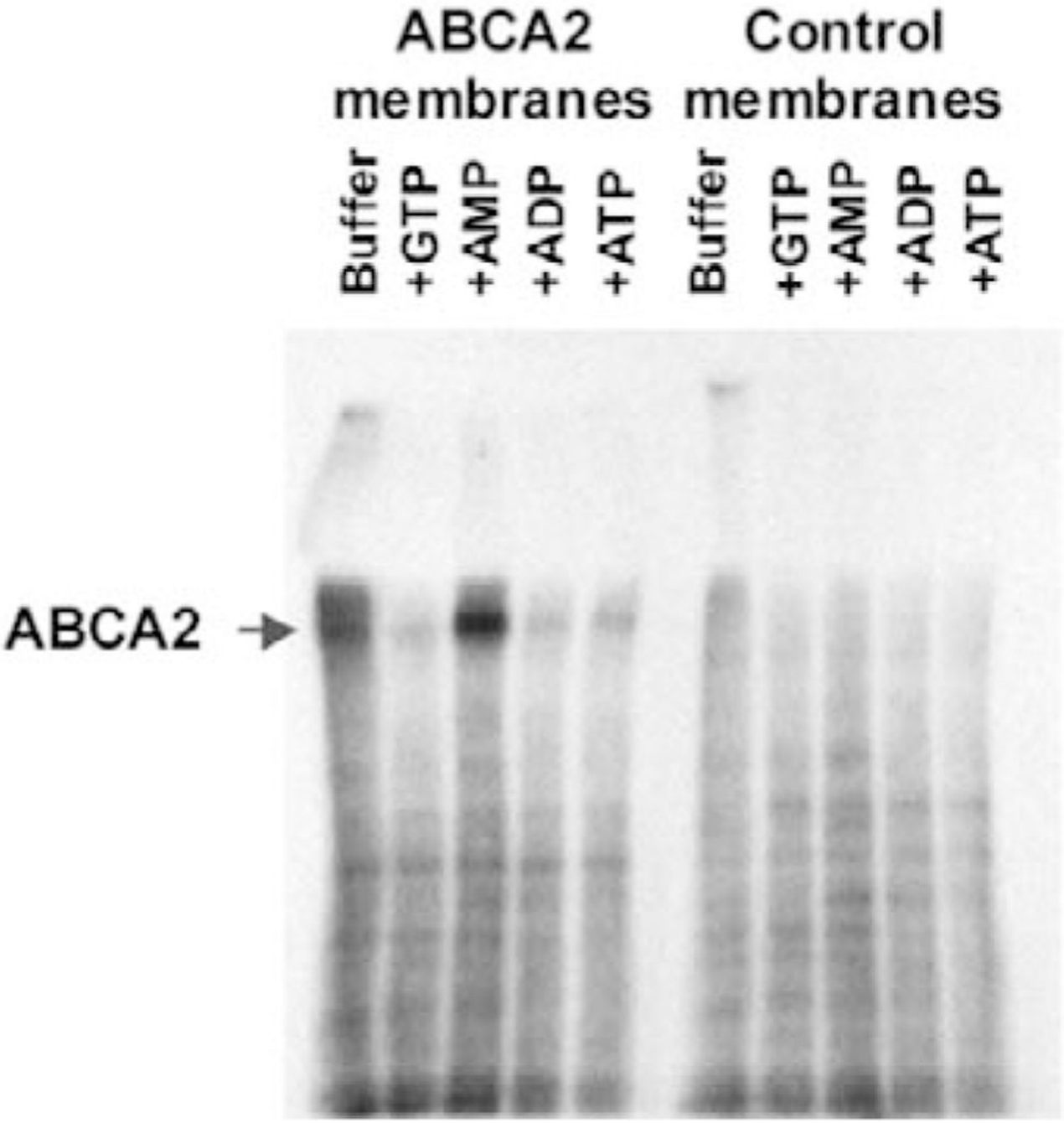

Figure 2.

Binding of pseudo-substrate, 8-azido-[α-^{32}P]-ATP, to a membrane-embedded ABCA2. Isolated membranes were incubated with 8-azido-[α-^{32}P]-ATP in the presence of potential competitors (ATP, ADP, GTP or AMP) at 0°C for 10 min and exposed to UV light for 10 min, separated by 6% SDS-PAGE, and exposed to film. The panel on the right displays an identical experiment performed with mock-transfected cells.

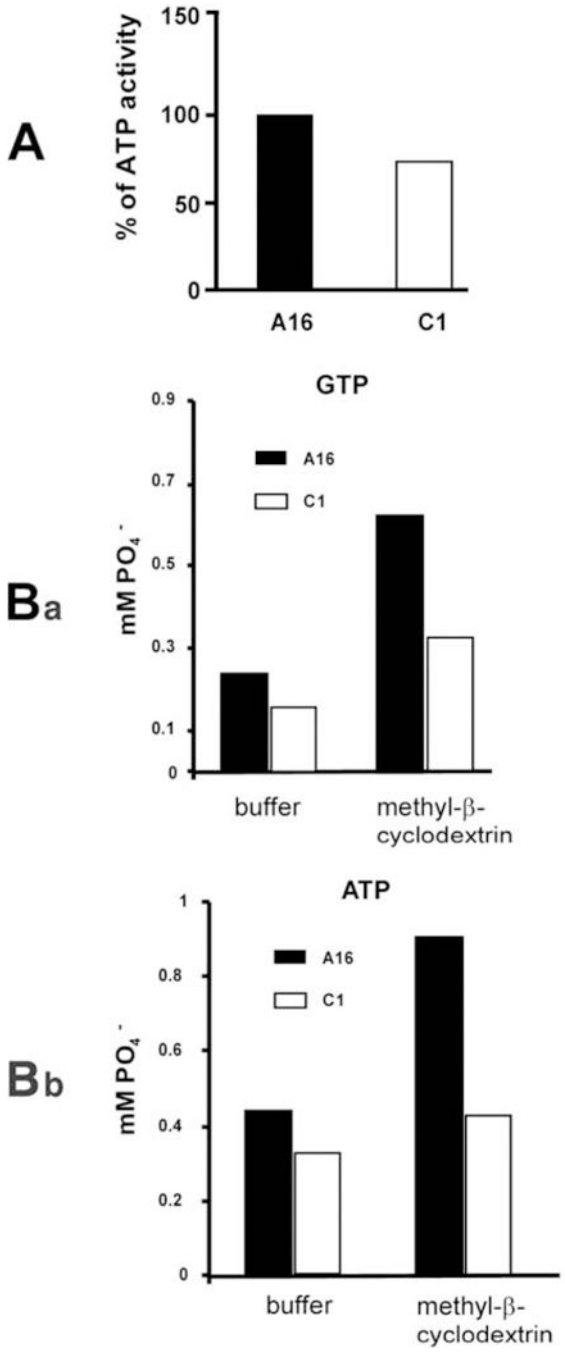

Figure 3.

ATPase activity of membranes isolated from ABCA2 transfected cells (A16) and mock-transfected cells (C1) gauged by the release of PO_4^{3+} ions. A) Bar graphs displaying the relative ATPase activity of the cells transfected with ABCA2 and mock-transfected cells. B) Stimulation of the ATPase and GTPase activity of ABCA2 by the addition of the methyl-β-cyclodextrin. Standard errors were less than 10% of the mean value and are not shown.

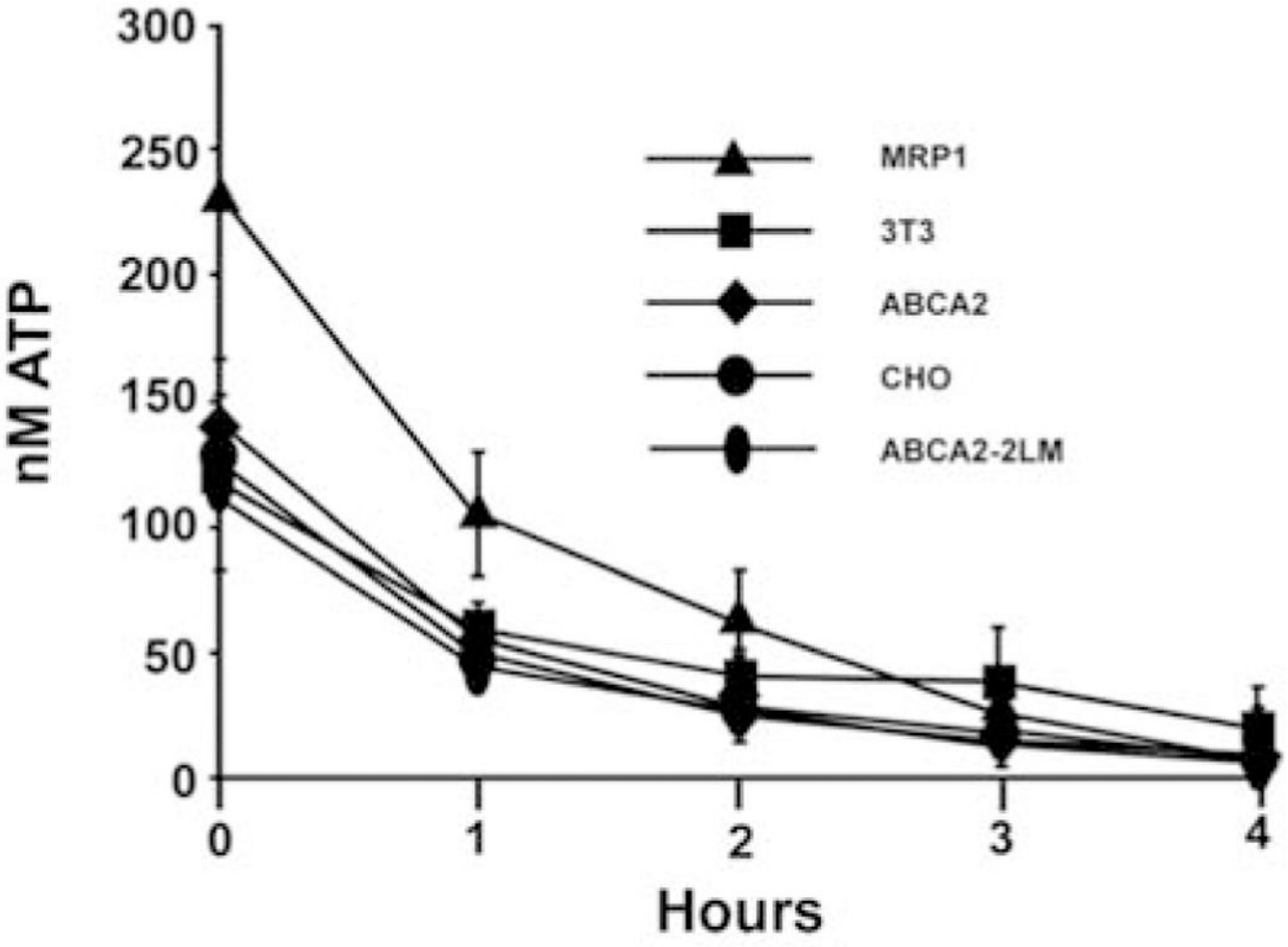

Figure 4.

Hydrolysis of endogenous ATP by cells transfected with MRP1, ABCA2 and ABCA2-2LM. Cells transfected with empty vectors, NIH/3T3 and CHO, are included. Intracellular concentrations of ATP were determined by the luciferase assay.