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Protein kinase C regulates amino acid transporter ATB$^{0,+}$

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ABSTRACT

ATB$^{0,+}$ (*SLC6A14*) is a transporter specific towards neutral and cationic amino acids, known to be up-regulated in malignant tumor cells. We cloned cDNA for rATB$^{0,+}$ and expressed it in HEK 293 cells. The ATB$^{0,+}$ over-expression correlated with increased L-leucine transport, stimulated by protein kinase C (PKC) activator and attenuated by PKC inhibitors. Transport stimulation was correlated with phosphorylation on serine moiety of the transporter and its augmented plasma membrane presence. Immunoprecipitation experiments demonstrated ATB$^{0,+}$ interaction with PKCα, but not with other classical or novel PKC isoforms. Immunocytochemistry experiments showed a transfer of PKCα to plasma membrane upon phorbol ester activation and co-localization with ATB$^{0,+}$. The observed regulation of ATB$^{0,+}$ by PKC correlates with high activity of both proteins reported for cancer cells.

1. Introduction

Amino acid transporter ATB$^{0,+}$-solute carrier family 6 member 14 (*SLC6A14*), specific towards a broad spectrum of cationic and neutral amino acids [1] and capable of transporting carnitine with a low affinity [2], is highly expressed in malignant cell lines, where it can be up-regulated by estrogen receptor, what makes it a potential target for cancer chemotherapy [3,4]. ATB$^{0,+}$ belongs to a superfamily of amino acids, neurotransmitters and osmolytes transporters catalyzing symport of 1 substrate molecule with 2 Na^+ and 1 Cl^- and probably having 12 transmembrane domains with a proposed cytoplasmic localization of the N- and C-termini [5]. Several members of this superfamily have been reported to be regulated by protein kinase C (PKC), whose activation correlated with decreased activity and internalization of transporter protein [6–12].

Recently, it was found, however, that, on the contrary to other members of solute carrier family 6, treatment of astrocytes with PKC activator increased ATB$^{0,+}$surface expression and leucine accumulation [13] – a process which could also depend on activity of system L-LAT transporters, specific towards neutral amino acids [14]. Due to the similar sensitivity of system L and ATB$^{0,+}$ to the same inhibitor (2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid) and an overlapping substrate specificity, as well as functioning of other transporters specific for cationic amino acids in the cell, it was difficult to ascribe the observed phenomena to a direct effect on ATB$^{0,+}$ itself. Therefore, we heterogously expressed ATB$^{0,+}$ with a C-terminally tagged 3×FLAG peptide and the present study was focused on a possible direct regulation by PKC, in particular on ATB$^{0,+}$ phosphorylation, localization, transport activity and interaction with particular PKC isoforms.

2. Materials and methods

2.1. Antibodies and chemicals

The polyclonal antibody against rat ATB$^{0,+}$ was raised by GenScript Corporation, as given in [13]. PKCα antibody was from Cell Signaling, while other anti-PKC antibodies and a monoclonal anti-phosphoserine antibody (clone 4A9) were from Alexis Biochemicals (Enzo Life Sciences). Alexa Fluor 488 labeled anti-rabbit and Alexa Fluor 555 anti-mouse antibodies were from Molecular Probes. EZ-Link® Sulfo-NHS-LC-Biotin [Sulfosuccinimidyl-6-(biotinamido)hexanoate] and Pierce® Avidin Agarose Resin were from Pierce. Leucine, L-[3,4,5-^{3}H(N)] was from NEN Life Science products. Monoclonal anti-FLAG M2 antibody, anti-FLAG M2 affinity agarose gel, succinyl-concanavalin A-FITC labeled and all other reagents were from Sigma.

2.2. Plasmid construction

RNA was isolated from rat lungs with TRIzol [15], subjected to reverse transcription-PCR with Enhanced Avian HS RT-PCR kit

Abbreviations: Bis II, bis-indolylmaleimide II; DMSO, dimethylsulfoxide; FITC, fluorescein isothiocyanate; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole; LAT, system L amino acid transporter; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate.

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(Sigma). Gene of rATB$^{0,+}$ (accession No. NCBI EF494736) was amplified with the primers 5′-TAAAGCTTATGGACAGATTGAAGT-3′ and 5′-TAGGATCCTTCTGGTTTTCTAGTGCC-3′ (introduced recognition sites for HindIII and BamHI sites respectively are underlined) with annealing temperature of 53.3 °C. Reamplification with Phusion polymerase (Finnzymes) was performed with the primers 5′-AAGCTTATGGACAGATTG-3′ and 5′-GGATCCTTCTGGTTTTC-3′ with annealing temperature of 56 °C. Product was cloned into SmaI site of pBluescript II KS (+) vector. Insert was recloned as HindIII–BamHI fragment in p3×FLAG–CMV14 plasmid. Resulting plasmid (p3×FLAG–CMV14/B$^{0,+}$) express ATB$^{0,+}$ with C-terminal FLAG fusion. All plasmid constructs were verified by sequencing.

2.3. Cell culture

Transfection of HEK 293 cells with either p3×FLAG–CMV14 or p3×FLAG–CMV14/B$^{0,+}$ plasmids was performed with Lipofectamine™ 2000 (Invitrogen), according to supplier protocol. The cells were cultured in 10% fetal bovine serum, 90% Minimal essential medium with Earle's salts, supplemented with 2 mM glutamine, G418 (300 μg/ml), gentamycin 50 μg/ml and fungizone (0.25 μg/ml) at 37 °C in a humid atmosphere of 5% CO_2. Before the experiment the cells were washed with 138 mM NaCl, 10 mM Tris, pH 7.4 and treated either with 0.4% dimethylsulfoxide (DMSO) as vehicle or either with 10 μM bis-indolylmaleimide II (Bis II) or 10 μM Gö 6976 for 5 min, followed by 30 min treatment with 200 nM (if not otherwise stated) phorbol 12-myristate 13-acetate (PMA). Preincubation with all the compounds added alone lasted either for 35 min in case of inhibitors or 30 min for PMA.

2.4. Immunoprecipitation and immunoblotting

Cells were lysed in 150 mM NaCl, 50 mM Tris, pH 7.4 supplemented with 1% NP-40, protease inhibitor cocktail (Fermentas) and phosphatase inhibitors (Roche) and after protein estimation subjected either to immunoblotting or to immunoprecipitation with anti-FLAG M2 affinity agarose gel. Elution was performed with either electrophoresis sample buffer for phosphorylation studies or with 3×FLAG® PEPTIDE (7.5 μg). The blots were analyzed with antibodies indicated in Figures and legends. The quantitative analysis of the bands intensity was performed with the INGENIUS apparatus (Syngen Biotech) with the software Gen Snap Ingenius Bioimaging for image registration and Gene Tools Ingenius Bioimaging for analysis.

2.5. Immunocytochemistry

Cells were washed three times with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at 4 °C. After three consecutive washes they were either directly subjected to blocking with 10% goat serum in PBS or subjected to permeabilization with 0.1% Triton X-100 in PBS before a blocking step. They were subsequently incubated either with FITC-conjugated-concanavalin A (100 μg/ml) and anti-FLAG antibody (1:1000) or with anti-PKCα (1:100) and anti-FLAG antibody. For detection of primary antibodies Alexa Fluor 488 labeled anti-rabbit and Alexa Fluor 555 anti-mouse antibodies were applied. Three-dimensional analysis was performed with the Inverted Leica DM IRE2 Confocal and Multiphoton Microscope, as given in [16].

2.6. Biotinylation of cell surface proteins

Biotinylation with EZ-Link® Sulfo-NHS-LC-Biotin and separation of biotinylated proteins with Pierce® Avidin Agarose Resin was performed as given in [13].

2.7. Transport assays

The culture medium was removed and the cell monolayer was washed twice with 138 mM NaCl, 10 mM Tris, pH 7.4. The cells were covered with the same buffer and, after treatment indicated in the Figure legends, transport of 90 nM L-leucine (7.2 Ci/mmol) was measured for 5 min at 37 °C. Transport was terminated by removal of incubation medium, two washes of the monolayer and two subsequent washes by centrifugation. The cells were solubilized in 1% SDS, 0.1 M NaOH, 2% Na_2CO_3 and the samples were taken for protein and radioactivity estimation. The mean ± SEM was calculated and the statistical analysis was performed using ANOVA with subsequent Bonferroni's multiple comparison test.

3. Results

3.1. Activation of ATB$^{0,+}$ by PKC

Out of a broad spectrum of ATB$^{0,+}$ substrates, the hydrophobic ones, especially isoleucine and leucine reveal the highest affinity [1]. Leucine transport was previously observed to be stimulated in rat astrocytes by PMA treatment [13] and, although part of this transport activation was reversed by chloride replacement, it was difficult to eliminate contribution of other systems capable of leucine accumulation. Therefore, the effect of PKC activator was verified in the present study, when comparing the cells transfected with either p3×FLAG–CMV14 plasmid alone or after rATB$^{0,+}$ overexpression upon transfection with p3×FLAG–CMV14/B$^{0,+}$. HEK 293 cells are capable of accumulating leucine (Fig. 1A), due to the presence of endogenous transporters, transfection with p3×FLAG–CMV14/B$^{0,+}$ increased transport of this amino acid by 30%. Taking into account that part of transporter protein can be localized inside the cell and, as observed previously in astrocytes, its surface presence can increase upon PKC activation, we verified the influence of PMA. As presented in Fig. 1A, PMA only slightly increased the basic leucine transport and strongly activated transport of this amino acid in p3×FLAG–CMV14/B$^{0,+}$ transfected cell, with the highest effect detected at 200 nM concentration. This activation by PMA was strongly inhibited and leucine transport activity was reversed to the basal control level by a general PKC inhibitor–Bis II [17], as well as by Gö 6976, known to be a specific inhibitor of the classical PKC isoforms [18] (Fig. 1B).

3.2. Interaction of ATB$^{0,+}$ with PKCa

Since we observed augmented leucine transport after PMA treatment, we checked for a possible interaction of ATB$^{0,+}$ with classical and novel PKC isoforms, known to be activated by phorbol esters [19]. As shown in Fig. 2, only the α isoform of PKC co-precipitated with ATB$^{0,+}$ after PMA treatment, neither other classical nor novel isoforms δ and ε could be detected. We further studied effect of PMA on localization of ATB$^{0,+}$ in the cells. It is interesting to note that there is no reaction with anti-FLAG antibodies, when cells were not permeabilized (Fig. 3A1), although the cells were labeled with concanavalin A reacting with sugar moieties present at the outer side of plasma membrane (Fig. 3A2 and A3). This result confirms a proposed topology of ATB$^{0,+}$ in the membrane with the C-terminus, tagged in a present system with 3×FLAG peptide, inside the cell. Under control conditions PKCα is dispersed in HEK 293 cells (Fig. 3B2 and D2) and it is transferred to plasma membrane upon PMA treatment (Fig. 3C2 and E2). In permeabilized HEK 293 cells transfected with p3×FLAG–CMV14/B$^{0,+}$ ATB$^{0,+}$ can be detected mainly in the cytoplasm (Fig. 3B1) and an increased amount of anti-FLAG reacting puncta was detected after PMA in plasma

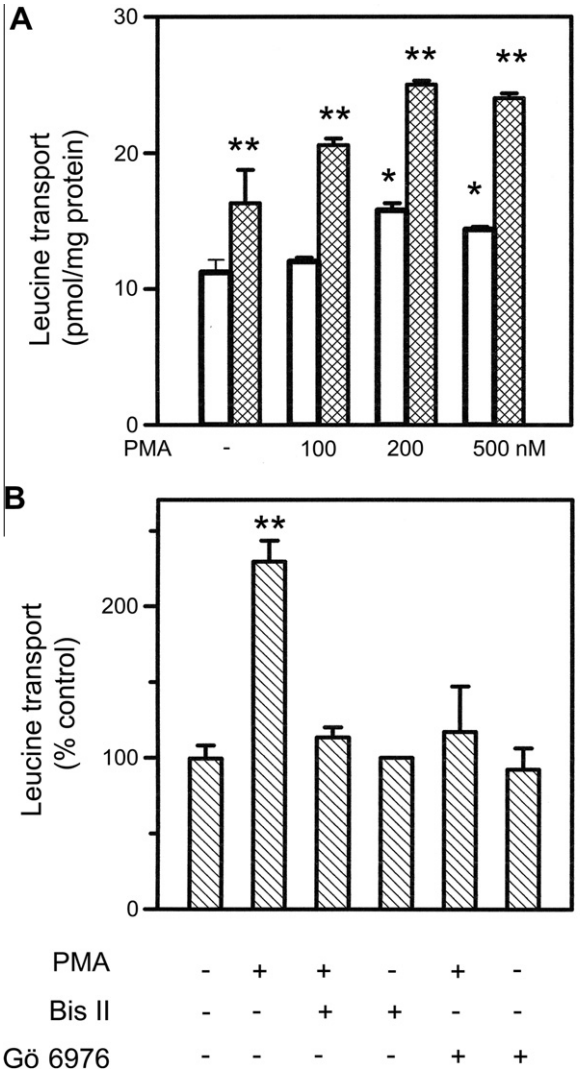

Fig. 1. Transport of L-leucine in HEK 293 cells after over-expression of $ATB^{0,+}$. (A) Effect of PMA on leucine transport in cells transfected with either p3×FLAG–CMV14 (open bars) or p3×FLAG–CMV14/$B^{0,+}$ (gray bars) plasmids, means ± SEM ($n = 9$). B. Effect of PMA and PKC inhibitors on leucine transport in p3×FLAG–CMV14/$B^{0,+}$ transfected cells, mean ± SEM ($n = 6$). Significance $^{*}p < 0.05$, $^{**}p < 0.001$.

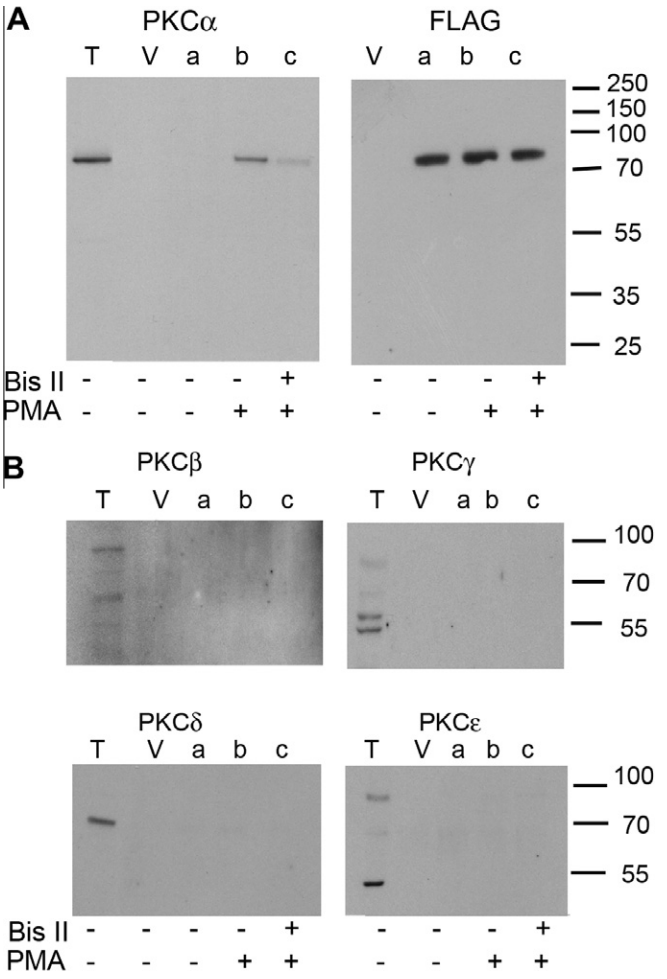

Fig. 2. Co-precipitation of PKC isoforms with $ATB^{0,+}$. HEK 293 cells transfected with p3×FLAG–CMV14/$B^{0,+}$ plasmid were incubated either with DMSO alone (lanes a) or with 200 nM PMA (lanes b), or Bis II and PMA (lanes c), as described in Section 2. The cells were lysed and the samples were either subjected to immunoblotting probed for an indicated PKC isoform (T), or subjected to immunoprecipitation with anti-FLAG M2 affinity agarose gel, V-indicates immunoprecipitation performed with lysate from the cells transfected with p3×FLAG–CMV14 alone. Elution was performed with 3×FLAG® Peptide and the eluate was analyzed for presence of either PKC isoforms or 3×FLAG tagged $ATB^{0,+}$. Blots are representative of 3 (A) or 2 (B) independent experiments.

membrane (Fig. 3C1). Partial co-localization with PKCα in plasma membrane was observed upon PMA treatment (Fig. 3C3).

3.3. Phorbol ester treatment augments $ATB^{0,+}$ phosphorylation and plasma membrane localization

The observed effect of 200 nM PMA could result either from phosphorylation of $ATB^{0,+}$ or from an indirect effect of phosphorylation of another protein. Therefore, the phosphorylation status of $ATB^{0,+}$ immunoprecipitated with anti-FLAG affinity gel was analyzed with an anti-phosphoserine antibody recognizing phosphoserine in vicinity of positively charged amino acids, a concensus site recognized by PKC [20]. As presented in Fig. 4A, electrophoresis sample buffer recommended for elution for phosphorylation analysis releases the antibody (indicated by an arrow). PMA treatment resulted in an increased phosphorylation of the FLAG-tagged transporter (a band indicated by an asterix) by 80 ± 14%, the effect reversed by Bis II pretreatment. There are as well some co-precipitating phosphorylated proteins of $M_r = 40{,}000$ and 30,000 detected in the blot, the identity of which has to be established, since, due the lack of reactivity with anti-FLAG antibodies, they cannot result from $ATB^{0,+}$ degradation. In order to verify if the observed increased phosphorylation of the band migrating with the same electrophoretic mobility as the one detected with anti-FLAG antibodies resulted in changed plasma membrane presence of the transporter, biotinylation with membrane non-permeable biotinylating reagent was performed, followed by homogenization and Avidin resin chromatography [13]. Proteins eluted from the avidin resin with sample buffer were analyzed for presence of $ATB^{0,+}$ and, as shown in Fig. 4B, the band migrating with $M_r = 60{,}000$ can be detected also in the cells transfected with p3×FLAG–CMV14 alone (V) and corresponds most probably to the endogenous $ATB^{0,+}$, moreover, its intensity does not change upon PMA treatment. On the contrary, the upper band (indicated by asterix) migrating with the same mobility as the one detected with an anti-FLAG antibody was almost invisible under control conditions and its amount significantly increased after PKC activation.

4. Discussion

Administration of PMA resulted in increased activity of leucine transport catalyzed by over-expressed $ATB^{0,+}$, what correlated with

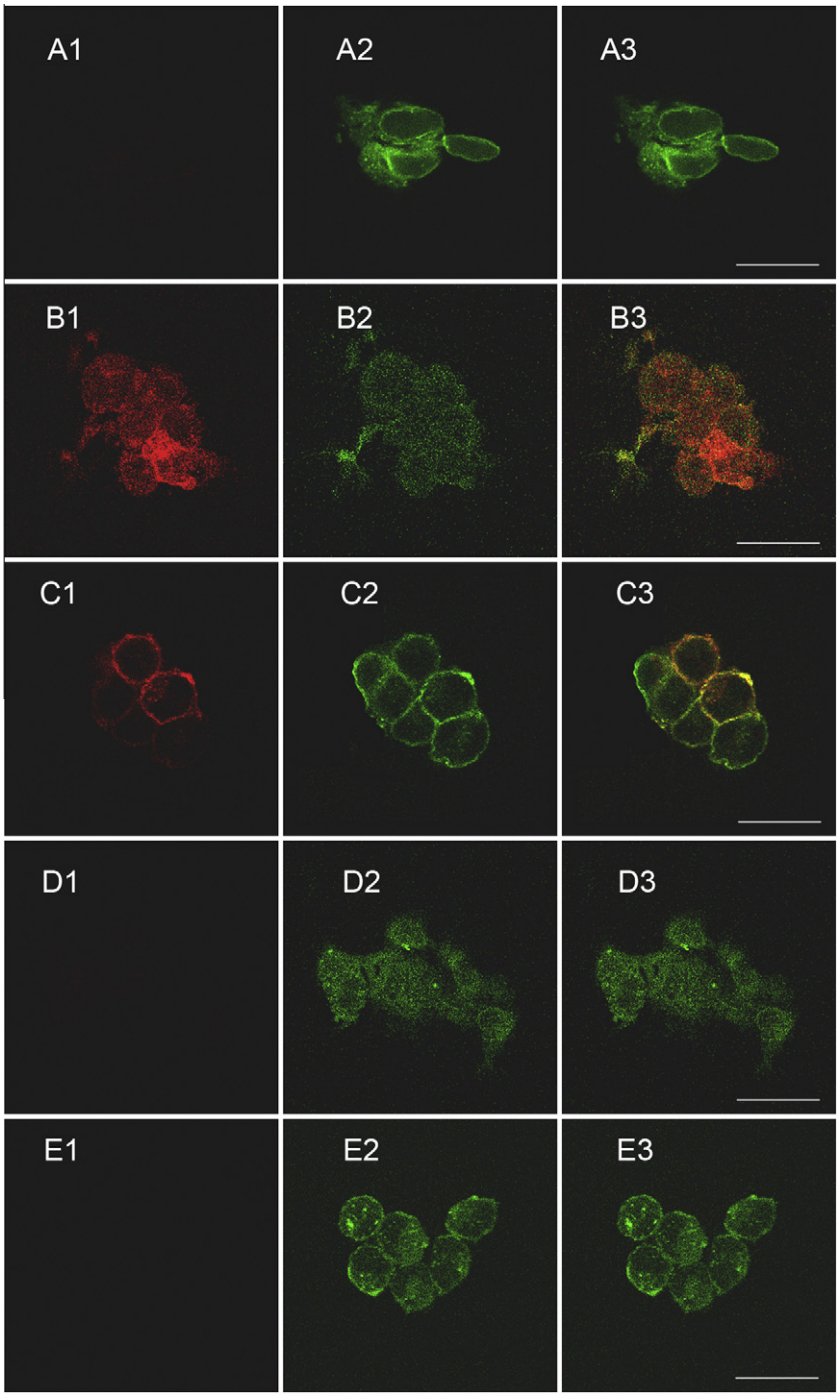

Fig. 3. Localization of $ATB^{0,+}$ in HEK 293 cells. HEK 293 cells transfected with p3×FLAG–CMV14/$B^{0,+}$ (A, B and C) or with p3×FLAG–CMV14 (D and E) were analyzed for presence of over-expressed $ATB^{0,+}$ with anti-FLAG antibodies (panels with indices 1), or PKCα (panels B2, C2, D2, E2). Panels A1, A2, A3 show analysis of non-permeabilized cells visualized by presence of externally reacting concanavalin A. Panels with indices 3 show the merged images. The representative slices from confocal microscope analysis are shown. Bar 25 μm.

an increased amount of transporter in plasma membrane. Although phorbol esters are known to bind to some other than PKC proteins, as chimaerins, Munc13 proteins and Ras guanyl-releasing proteins [21], the increased phosphorylation of $ATB^{0,+}$ on serine moiety, transporter co-precipitation with PKCα and increased co-localization of both proteins in plasma membrane as well as sensitivity of the observed effects to PKC inhibitor–Bis II, suggest a direct regulation of $ATB^{0,+}$ by PKC, what seems especially interesting in terms of high expression of $ATB^{0,+}$ in malignant cell lines. It should be also added that PKC activators, as phorbol esters, have been known as tumor-promoting agents [22] and PKC-dependent signaling pathways have been reported to control many cellular functions, including proliferation. As demonstrated in biotinylation experiments, endogenous $ATB^{0,+}$ can be detected in plasma membrane, anyhow, its amount does not change upon PMA treatment. This could suggest that plasma membrane subpopulation of the transporter is already phosphorylated. Co-localization of over-expressed $ATB^{0,+}$ with PKCα at plasma membrane

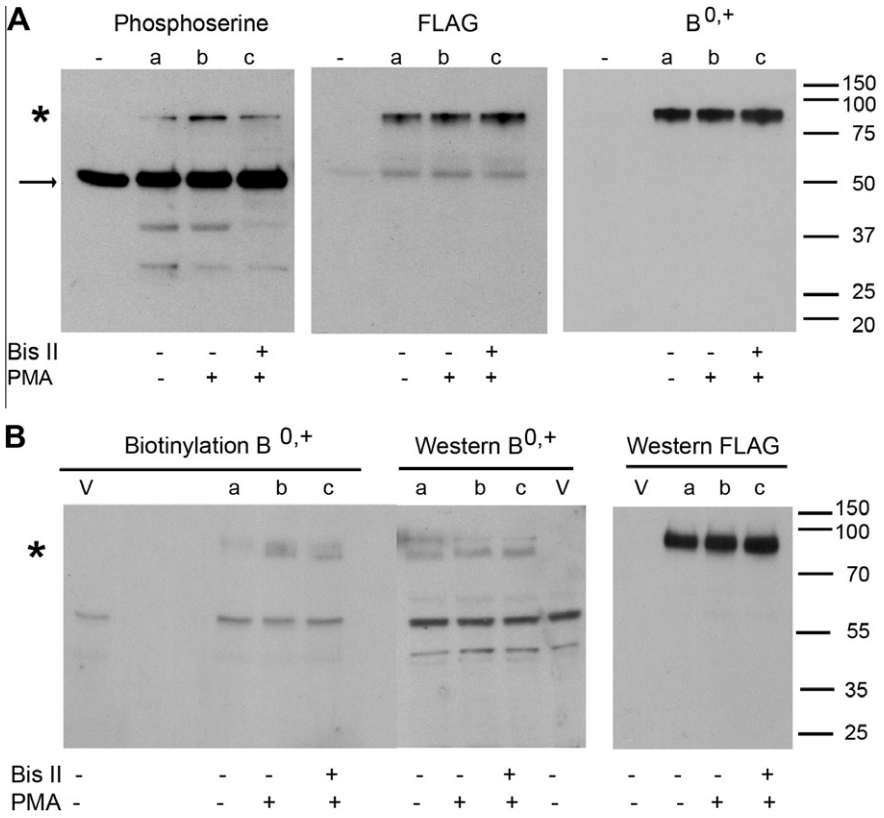

Fig. 4. Effect of PMA on $ATB^{0,+}$ phosphorylation and surface presence. HEK 293 cells transfected with p3×FLAG–CMV14/$B^{0,+}$ plasmid were incubated either with DMSO (lanes a) alone or with 200 nM PMA (lanes b), or Bis II and PMA (lanes c), as described in Section 2. (A) Phosphorylation analysis: The cells were lysed and the samples were subjected to immunoprecipitation with anti-FLAG M2 affinity agarose gel and analyzed for presence of phosphoserine, $B^{0,+}$ and FLAG. The left lane (−) shows treatment of the gel with the lysis buffer alone. Arrow shows unbound antibody eluted with electrophoresis sample buffer, the asterix–FLAG-tagged $ATB^{0,+}$. (B) Analysis of cell surface biotinylated proteins and Western blot analysis of $ATB^{0,+}$ and FLAG-tagged protein in either p3×FLAG–CMV14/$B^{0,+}$ (a, b and c) or p3×FLAG–CMV14-transfected cells (V). Blots are representatives of three independent experiments.

suggests that there is another PKC-dependent mechanism leading to transporter trafficking to the surface, a process most probably dependent on activation of other protein(s). Recently, it was shown that PKC-dependent activation of ubiquitination enzyme led to an opposite process, an increased endocytosis of a glutamate transporter GLT-1 [23]. On the other hand, an increased surface presence of SLC6 family members depends on their interaction with Sec24 proteins [24,25]. The mechanism of $ATB^{0,+}$ trafficking to the cell surface has been still obscure and the indentity of protein(s) responsible for $ATB^{0,+}$ movement to plasma membrane and the PKC-dependence of this process remain to be established.

It has been quite surprising that the observed effects of PKC on $ATB^{0,+}$ are opposite to the effects described in case of other members of SLC6 family. This might be caused by different physiological roles, since neurotransmitter transporters catalyze re-uptake and have to respond to a high substrate concentration outside the cell, while $ATB^{0,+}$ delivers amino acids to the cell in response to nutritional demand of the cell. It has to be added that, due to its broad substrate specificity (including NO precursor–arginine) $ATB^{0,+}$ can supply the necessary amino acids for quickly proliferating cells.

Therefore, both targeting $ATB^{0,+}$ with specific inhibitors [3], as well as understanding of this transporter regulation in the cell can have important medical implications.

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