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Activation of classical protein kinase C reduces the expression of human cationic amino acid transporter 3 (hCAT-3) in the plasma membrane

Alexander ROTMANN*[1], Nicole VÉKONY*[1], Davina GASSNER*, Günter NIEGISCH*, Dennis STRAND†, Ursula MARTINÉ* and Ellen I. CLOSS*[2]

*Department of Pharmacology, Johannes Gutenberg University, Obere Zahlbacher Strasse 67, 55101 Mainz, Germany, and †First Department of Internal Medicine, Johannes Gutenberg University, Obere Zahlbacher Strasse 63, 55101 Mainz, Germany

We have previously shown that activation of PKC (protein kinase C) results in internalization of hCAT-1 [human CAT-1 (cationic amino acid transporter 1)] and a decrease in arginine transport [Rotmann, Strand, Martiné and Closs (2004) J. Biol. Chem. **279**, 54185–54192]. However, others found increased transport rates for arginine in response to PKC activation, suggesting a differential effect of PKC on different CAT isoforms. Therefore we investigated the effect of PKC on hCAT-3, an isoform expressed in thymus, brain, ovary, uterus and mammary gland. In *Xenopus laevis* oocytes and human U373MG glioblastoma cells, hCAT-3-mediated L-arginine transport was significantly reduced upon treatment with compounds that activate classical PKC. In contrast, inactive phorbol esters and an activator of novel PKC isoforms had no effect. PKC inhibitors (including the PKCα-preferring Ro 31-8280) reduced the inhibitory effect of the PKC-activating compounds. Microscopic analyses revealed a PMA-induced reduction in the cell-surface expression of fusion proteins between hCAT-3 and enhanced green fluorescent protein expressed in *X. laevis* oocytes and glioblastoma cells. Western-blot analysis of biotinylated surface proteins demonstrated a PMA-induced decrease in hCAT-3 in the plasma membrane, but not in total protein lysates. Pretreatment with a PKC inhibitor also reduced this PMA effect. It is concluded that similar to hCAT-1, hCAT-3 activity is decreased by PKC via reduction of transporter molecules in the plasma membrane. Classical PKC isoforms seem to be responsible for this effect.

Key words: arginine, classical protein kinase C, human cationic amino acid transporter 3 (hCAT-3), plasma membrane, PMA, system y^+.

INTRODUCTION

In most cell types, a significant part of cationic amino acid transport is mediated by the so-called system y^+ first described in fibroblasts (for a review, see [1]). System y^+ is characterized by selectivity for cationic amino acids, half maximal activity at cationic amino acid concentrations (K_m) of 0.1–0.2 mM, Na^+- and pH-independence, and strong stimulation of transport by substrate at the *trans* side of the membrane (*trans*-stimulation). PKC (protein kinase C) activation has been reported both to stimulate and to down-regulate system y^+ activity, suggesting a differential action of PKC on different carrier proteins mediating system y^+ activity. Three such carrier proteins that belong to the family of CATs (cationic amino acid transporters) have been identified (for reviews, see [1,2]). Of these, CAT-1 seems to conform best with system y^+, as at least among the human isoforms [hCATs (human CATs)], hCAT-1 shows the highest apparent affinity, the strongest *trans*-stimulation and independence of pH changes over a wide range. We have previously shown that hCAT-1-mediated transport is down-regulated in both *Xenopus laevis* oocytes and human endothelial cells upon PKC activation [3]. This reduction in transport activity is not accompanied by a decrease in the total level of hCAT-1 protein. Others confirmed these findings in porcine pulmonary artery endothelial cells and identified cPKC (classical PKC) isoforms, most likely PKCα, as responsible for this down-regulation [4]. In a recent study, we demonstrated that the PKC-mediated inhibition of hCAT-1 is due to a reduction of transporter protein in the plasma membrane [5].

In the present study, we investigated the effect of PKC activation on the hCAT-3 isoform. CAT-3 was first described in mice and rats. While it exhibits a wide expression pattern in embryonal mouse tissues, CAT-3 seems to be confined to the brain in adult mice and rats [6,7]. This is in contrast with the almost ubiquitous expression of CAT-1 (for a review, see [8]). A special role of CAT-3 providing substrate for the nNOS (neuronal nitric oxide synthase) has been proposed based on the neuron-specific expression of CAT-3 in rat brain [9]. However, in humans, hCAT-3 exhibits a much wider expression pattern that does not correlate with the expression pattern of nNOS [10]. The strongest hCAT-3 expression has been observed in thymus and a weaker expression in brain, ovary, uterus and mammary gland. Although hCAT-3 has a slightly lower apparent substrate affinity and is less dependent on *trans*-substrate than hCAT-1, it would be difficult to distinguish the two isoforms when expressed in the same cell. hCAT-3 was thus a good candidate for a system y^+ transporter that might be differentially regulated by PKC. The effect of PKC activation on hCAT-3 overexpressed in *X. laevis* oocytes and the human glioblastoma cell line U373MG was therefore investigated.

EXPERIMENTAL

Transport studies in *X. laevis* oocytes

The constructs HC3.pSP64T and hCAT-3.EGFP-pSP64T encoding respectively wild type hCAT-3 and hCAT-3 fused to the N-terminus of EGFP [enhanced GFP (green fluorescent protein)]

Abbreviations used: 4α-PDD, 4α-phorbol-12,13-didecanoate; BIM I, bisindolylmaleimide I; GFP, green fluorescent protein; EGFP, enhanced GFP; GABA, γ-aminobutyric acid; GAT1, GABA transporter 1; GST, glutathione S-transferase; CAT, cationic amino acid transporter; hCAT, human CAT; nNOS, neuronal nitric oxide synthase; PDBu, phorbol-12,13-dibutyrate; PIP, L-α-phosphatidylinositol-3,4,5-trisphosphate-dipalmitoyl; PKC, protein kinase C; aPKC, atypical PKC; cPKC, classical PKC; nPKC, novel PKC; PNGase F, peptide N-glycosidase F; trpE, tryptophan E.

[1] These authors have contributed equally to this work.

[2] To whom correspondence should be addressed (email Closs@mail.uni-mainz.de).

have been described earlier [10]. They were linearized with SalI and AflIII respectively and cRNA was prepared by *in vitro* transcription from the SP6 promoter (mMessage mMachine *in vitro* transcription kit; Ambion, AMS Biotechnology Europe, Cambridgeshire, U.K.). cRNA (30 ng in 40 nl of water) was injected into each *X. laevis* oocyte (Dumont stage V–VI). Oocytes injected with 40 nl of water were used as control.

Arginine uptake was determined 2 days after injection of cRNA as described previously [11]. Briefly, oocytes were incubated for 30 min at 20 °C in uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Hepes and 5 mM Tris, pH 7.5) containing 1 mM unlabelled arginine and as indicated 100 nM PMA, or 1 μM BIM I (bisindolylmaleimide I) plus 100 nM PMA. BIM I was added 5 min before PMA and control cells were incubated in 0.2 % DMSO. Oocytes were then washed in uptake solution containing 1 mM arginine and transferred to the same solution supplemented with 5 μCi/ml L-[^{3}H]arginine (ICN, Eschwege, Germany; L-[4,5-^{3}H]arginine; 39 Ci/mmol). After incubation for 15 min at 20 °C, oocytes were washed four times in ice-cold uptake solution and solubilized individually in 2 % (w/v) SDS. The incorporated radioactivity was determined in a liquid-scintillation counter.

Cryostat sections of *X. laevis* oocytes

Two days after injection with hCAT-1.EGFP cRNA, oocytes were treated with 100 nM PMA or 0.1 % DMSO alone (30 min, 20 °C) in uptake solution containing 1 mM arginine. The oocytes were then washed five times in PBS and immediately frozen in liquid nitrogen in a Tissue Freezing Medium (Leica Instruments, Benzheim, Germany). Cryostat sections (12 μm) were made at − 15 °C, and fluorescent micrographs were taken using a Leitz DMRB fluorescence microscope (Leica) and a Nikon DXM1200 digital camera (Nikon, Tokyo, Japan).

Cell culture

The human U373MG glioblastoma cells were obtained from A.T.C.C. (Manassas, VA, U.S.A.). Cells were grown in IMDM [Iscove's modified Eagle's medium, constituents as supplied by PAA Laboratories (Cat. No. E15-819; Cölbe, Germany)] supplemented with 10 % (v/v) heat-inactivated fetal bovine serum. U373MG-derived clones stably expressing hCAT-3 fused to the N-terminus of EGFP and described previously [10] were maintained in media containing 600 μg/ml G418. The human testis teratocarcinoma cell line NT2 was purchased from Stratagene (Heidelberg, Germany). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 [1:1 nutrient mix, constituents as supplied by PAA Laboratories (Cat. No. E15-813)], supplemented with 10 % fetal bovine serum. Cells were regularly tested for mycoplasma infection using DAPI (4,6-diamidino-2-phenylindole; Roche Molecular Biochemicals, Mannheim, Germany). No contamination was detected.

Transport studies in human U373MG glioblastoma cells

Cells grown to confluence in 96-well plates were preincubated for the indicated times at 37 °C in 100 μl/well of medium containing PMA or other compounds at the concentrations given in the Figure legends. The cells were then washed once with Locke's solution (154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 3.6 mM $NaHCO_3$ and 5.6 mM glucose, pH 7.4) containing 100 μM arginine, before incubation in the same solution containing in addition 5–10 μCi/ml L-[^{3}H]arginine (39 Ci/mmol) for 30 s at 37 °C. The cells were then immediately transferred to ice, washed three times with ice-cold Locke's solution and lysed in 0.5 M NaOH (50 μl/well, 30 min at 4 °C). After neutralization of the lysates with 50 μl of 0.5 M HCl and 100 μl of buffer A (50 mM Tris/HCl, pH 7.4, 0.5 mM EDTA and 0.5 mM EGTA), the protein content of each sample was determined using the Bradford reaction (Bio-Rad, Munich, Germany). The radioactivity in the samples was measured by liquid-scintillation counting. Values from cells stably transfected with pEGFP-N1 [average: 1.7 ± 0.4 pmol $\cdot$ (μg of protein)$^{-1} \cdot$ min^{-1}] were subtracted from the values obtained from hCAT-3.EGFP-expressing cells.

Confocal microscopy

Cells were grown on glass bottom chamber slides (Nalge Nunc, Naperville, IL, U.S.A.) and analysed with a Zeiss 510 confocal laser scan microscope equipped with a UV laser (Zeiss, Oberkochem, Germany). Images were collected with a 1.4 numerical aperture 63 × Zeiss Plan-aprochromat objective using identical scanning parameters.

Generation of an immune plasma specific for hCAT-3

A cDNA fragment coding for the 42 C-terminal amino acids of hCAT-3 was cloned in frame into pATH-1, 3′ to the coding region of trpE (tryptophan E) [12]. The resulting plasmid was transfected into Epicurian Coli™ XL1-Blue cells (Stratagene). Expression of the trpE–hCAT-3 fusion protein was induced by growth in tryptophan-free M9 medium containing 10 μg/ml 3,β-indoleacrylic acid (Sigma, Deisenhofen, Germany) (4 h, 37 °C). Bacteria were lysed by sonication in HEMGN buffer (100 mM KCl, 25 mM Hepes, pH 7.6, 0.1 mM EDTA, 12 mM $MgCl_2$, 10 % (v/v) glycerol and 0.1 % Nonidet P40) containing 1 mM dithiothreitol, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM PMSF, 0.2 mM $NaHSO_3$ and 0.5 mg/ml lysozyme. The lysates were spun at 26000 ***g*** and the supernatants (~ 15 mg of protein) were separated by SDS/PAGE (12.5 % gel). After Coomassie Blue staining, the gel portion containing the fusion protein was cut out and homogenized in 1 ml of water/g of gel using a 26 gauge needle. Six-week-old rabbits were immunized with 300 μl of homogenate (250 μg of fusion protein) and an equal volume of complete Freund's adjuvant (Life Technologies, Eggenstein, Germany). The immune plasma was collected after boosting the rabbits three times (every 3 weeks) with 250 μg of fusion protein (in 300 μl) and 1 vol. of incomplete Freund's adjuvant.

Affinity purification of immune plasma to hCAT-3

A cDNA fragment coding for the 42 C-terminal amino acids of hCAT-3 was cloned in frame into pGEX-3X, 3′ to the coding region of GST (glutathione S-transferase) [13]. The resulting plasmid was transfected into Epicurian Coli™ XL1-Blue cells (Stratagene). Expression of the GST–hCAT-3 fusion protein was induced by growth in LB (Luria–Bertani) medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside (Roche Molecular Biochemicals; 5 h, 37 °C). Bacteria were lysed on ice by sonication in PBS (2.7 mM KCl, 140 mM NaCl, 1.8 mM KH_2PO_4 and 10 mM Na_2HPO_4). After addition of 0.1 % Triton X-100, the lysates were spun at 7500 ***g*** and the fusion protein in the supernatants was purified using *S*-glutathione–Sepharose [14]. GST–hCAT-3 fusion protein (8 mg) was coupled with 4 ml of Affigel 10 (Bio-Rad; 4 °C, 4 h) and packed into a Poly-Prep chromatography column (Bio-Rad). Immune plasma (2 ml) was heat-inactivated (30 min, 56 °C), diluted 1 + 1 with PBS and applied to this column. After washing with 12 ml of PBS, antibodies were eluted with 0.1 M glycine/HCl (pH 2.5) and 1 M NaCl, neutralized with 1/10 vol. of 1 M Tris/HCl (pH 8) and dialysed against PBS (4 °C, 15 h).

Biotinylation of cell-surface proteins

Biotinylation of cell-surface proteins was performed as described in [15] with slight modifications. Briefly, cells were grown to confluence in 10 cm dishes [coated with poly(L-lysine) (molecular mass 150–300 kDa; Sigma) in the case of NT2 cells], rinsed with ice-cold PBS containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ (PBS-Ca/Mg) and incubated in the same solution supplemented with 1 mg/ml sulphosuccinimidobiotin [EZ-Link™ sulphosuccinimidyl-2-(biotinoamido)ethyl-1,3-dithiopropionate; Pierce] for 20 min at 4 °C. Cells were then rinsed three times with PBS-Ca/Mg containing 100 mM glycine and incubated in this buffer for 30 min at 4 °C to quench any unbound biotin. The cells were then lysed by the addition of 1 ml of RIPA (radioimmunoprecipitation assay) buffer (100 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS) containing protease inhibitors (Complete™; Roche) for 1 h at 4 °C. After removal of the cellular debris, protein concentration of the lysates was determined using the Bradford reaction. Biotinylated proteins were batch-extracted (from ~ 150 and 500 μg of total protein from U373 and NT2 cells respectively) using avidin-coated Sepharose beads (immobilized NeutrAvidin™, Pierce; 4 °C, overnight) and then released from the beads by incubation in 30 μl of sample buffer [50 mM Tris/HCl, pH 6.8, 2 mM EDTA, 4 % SDS, 8 M urea, 3.5 %, v/v, 2-mercaptoethanol and 0.001 % Bromophenol Blue; 30 min at room temperature (22 °C)]. To total cell lysates, an equal volume of sample buffer was added.

Western blots

Where indicated, lysates were treated with PNGase F (peptide N-glycosidase F; Roche; 2 units/100 μg of protein, 30 min at 37 °C). Total lysates and cell-surface proteins were separated in SDS/PAGE (10 % gel) and then blotted to nitrocellulose membranes (Protran83; Schleicher and Schuell, Dassel, Germany). Staining for EGFP fusion proteins and hCAT-proteins was achieved by sequential incubations in: Blotto [50 mM Tris/HCl, pH 8, 2 mM $CaCl_2$, 0.01 % antifoam A (Sigma), 0.05 % Tween 20 and 5 %, w/v, non-fat dry milk] containing 10 % goat serum (2 h, room temperature), a dilution of the first antibody in PBS containing 1 % bovine serum albumin and 0.1 % Tween 20 [1:500 of an anti-GFP peptide antibody (ClonTech, Heidelberg, Germany), or a 1:200 dilution of our hCAT-3 antibody, see above] (overnight, 4 °C), 3 × Blotto (15 min, room temperature), a 1:10 000 dilution in Blotto of a peroxidase-conjugated secondary goat anti-rabbit IgG antibody (Calbiochem, Bad Soden, Germany) (1 h, room temperature); 3 × TBST (10 mM Tris/HCl, pH 8, 150 mM NaCl and 0.05 % Tween 20), 1× in TBS (10 mM Tris/HCl, pH 8, and 150 mM NaCl) and finally for 1 min with chemiluminescence reagent (Renaissance; DuPont NEN, Bad Homburg, Germany). An X-ray film (Agfa, Leverkusen, Germany) was then immediately exposed to the membranes. Membranes were stripped (in 62.5 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS and 100 mM 2-mercaptoethanol; 30 min, 50 °C) and stained with a monoclonal antibody to β-tubulin (1:1000; Sigma) and a peroxidase-conjugated secondary goat anti-mouse IgG antibody (1:3000; Sigma).

RESULTS AND DISCUSSION

PMA-induced down-regulation of hCAT-3 activity in *X. laevis* oocytes

The effect of PKC activation was first studied in transport experiments with oocytes from *X. laevis* expressing hCAT-3. Treatment of the oocytes with 100 nM PMA led to a reduction in hCAT-3-mediated arginine transport down to 38.5 ± 4.7 % (Figure 1A). The PMA-induced reduction in arginine transport could be prevented by a concomitant treatment of the oocytes with the selective PKC inhibitor BIM I (1 μM) [16]. BIM I is a potent inhibitor of Ca^{2+}-dependent cPKCs (IC_{50} values *in vitro*: 8–20 nM) and a less potent inhibitor of nPKCs (novel PKCs) and aPKC (atypical PKC) isoforms (IC_{50} values *in vitro*: 210–700 nM and 5.8 μM respectively) [17]. This indicates that the PMA effect in our experiments was mediated by a classical or nPKC isoform expressed endogenously in *X. laevis* oocytes, such as PKC α, βI, βII, γ and ε [18,19]. To visualize the cell-surface expression of hCAT-3, EGFP was fused to the C-terminus of hCAT-3 and the fusion protein was expressed in the oocytes. Fluorescent micrographs revealed a discernible decrease of hCAT-3.EGFP expression in the oocyte plasma membrane upon PMA treatment (100 nM, 30 min; Figures 1B and 1C]. It seemed therefore that hCAT-3 responds to PKC activation in a similar way as hCAT-1 [5].

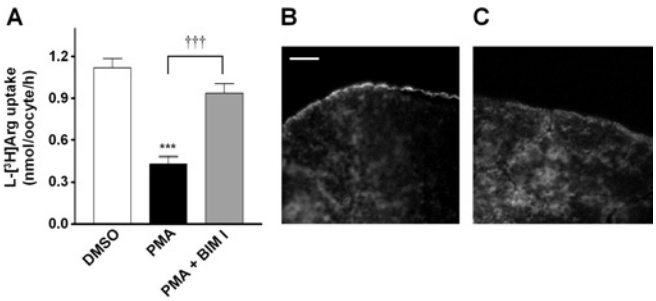

Figure 1 PKC-induced down-regulation of hCAT-3 in *X. laevis* oocytes

X. laevis oocytes were injected with cRNA encoding hCAT-3 (**A**) or hCAT-3.EGFP (**B**, **C**) and analysed 2 days later. (**A**) Uptake of 1 mM L-[^{3}H]arginine in oocytes treated for 30 min as indicated with 0.1 % DMSO alone or with 100 nM PMA (alone or in combination with 1 μM BIM I, in 0.2 % DMSO). Values obtained with water-injected oocytes (0.02 nmol · oocyte^{-1} · h^{-1}) were subtracted. Statistical analysis was performed using ANOVA with the Bonferroni post hoc test (***$P < 0.001$, compared with DMSO control; †††$P < 0.001$ between cells treated with PMA only and with PMA plus BIM I). (**B**, **C**) Fluorescent micrographs of cryosections from oocytes treated for 30 min with 0.1 % DMSO alone (**B**) or with 100 nM PMA in 0.1 % DMSO (**C**). The scale bar represents 50 μm.

Down-regulation of hCAT-3 activity by cPKC in human glioblastoma cells

Next, we expressed the hCAT-3.EGFP fusion protein in the human glioblastoma cells U373MG. Transport studies measuring the initial uptake rate for 100 μM L-[^{3}H]arginine revealed a 58 ± 2.8 % reduction in hCAT-3.EGFP-mediated transport upon PMA treatment (Figure 2A). In contrast, forskolin (50 μM) and IBMX (isobutylmethylxanthine; 600 μM) had no effect on the arginine transport (results not shown). This indicates that similar to hCAT-1 [4], hCAT-3 activity is regulated by PKC, but not by cAMP- or cGMP-dependent pathways. As observed with hCAT-3 expressed in *X. laevis* oocytes, pretreatment with BIM I reduced the PMA effect significantly. However, the activity of hCAT-3 was unchanged when BIM I was administered in the absence of an active phorbol ester. PDBu (phorbol-12,13-dibutyrate), another PKC-activating phorbol ester, reduced the transport rate to a similar extent as PMA (down to 43 ± 12 %). In contrast, the inactive phorbol ester 4α-PDD (4α-phorbol-12,13-didecanoate) had no significant effect. These results support the notion that PKC is responsible for the observed reduction in hCAT-3 activity. The extent of transport inhibition was dependent on the PMA concentration with half maximal inhibition observed at

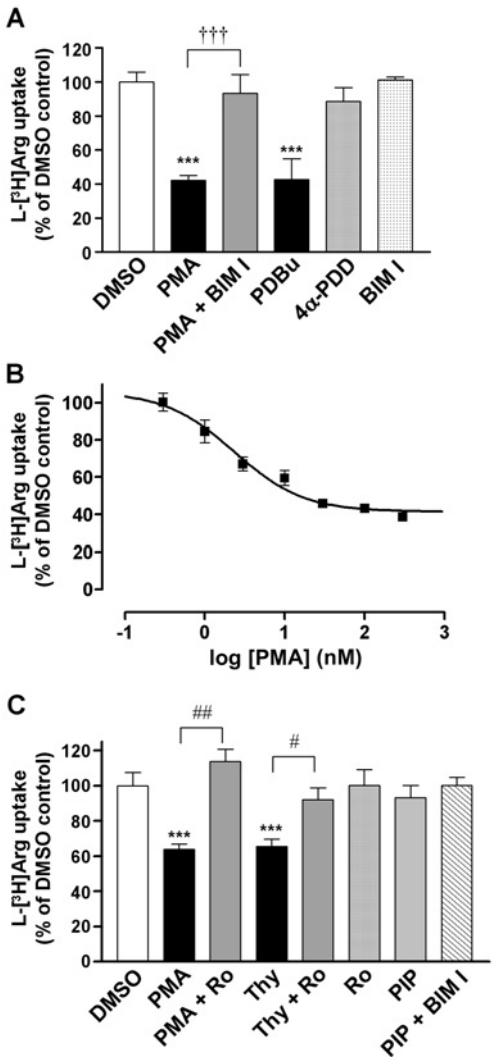

Figure 2 Inhibition of hCAT-3.EGFP-mediated arginine transport by cPKC in human U373MG glioblastoma cells

Uptake of 100 μM L-[^{3}H]arginine was measured in confluent U373MG glioblastoma cells stably transfected with hCAT-3.EGFP and seeded in 96-well plates. Immediately before the transport assay, cells were first treated for 15 min with 1 μM BIM I or 1 μM Ro 31-8220 [columns with BIM I (**A**) and Ro (**C**) respectively] or 0.1 % DMSO alone (all other columns; **A**, **C**) and then for further 30 min as indicated: (**A**) with 0.15 % DMSO alone, 100 nM PMA (alone or in combination with 0.5 μM BIM I), 100 nM PDBu, 100 nM 4α-PDD or 0.5 μM BIM I; (**B**) with 0.3–300 nM PMA; and (**C**) with 0.15 % DMSO alone, 100 nM PMA (alone or in combination with 0.5 μM Ro 31-8220), 100 nM thymeleatoxin (Thy) alone or in combination with 0.5 μM Ro 31-8220, 0.5 μM Ro 31-8220 and 5 μM PIP (alone or in combination with 0.5 μM BIM I). Values obtained with U373MG cells stably transfected with pEGFP-N1 were subtracted [1.7 ± 0.4 pmol $\cdot$ (μg of protein)$^{-1} \cdot$ min^{-1}]. Results are expressed as percentage of the transport rate of DMSO-treated cells [5.5 ± 0.3 pmol $\cdot$ (μg of protein)$^{-1} \cdot$ min^{-1}] (means $\pm$ S.E.M., $n = 30$–45). Statistical analysis was performed using ANOVA with the Bonferroni post hoc test (***$P < 0.001$, compared with DMSO control; †††$P < 0.001$ between cells treated with PMA only and with PMA plus BIM I; ## and #, P value < 0.01 and < 0.05 respectively between cells treated with PMA or thymeleatoxin alone and in combination with Ro 31-8220).

4.6 ± 1.3 nM PMA (Figure 2B). This is within the range of EC_{50} values determined for PMA-induced activation of PKC [20].

The PMA effect could also be prevented by Ro 31-8220 (1 μM), which inhibits preferentially Ca^{2+}-dependent cPKC isoforms (Figure 2C). In addition, thymeleatoxin (100 nM), a selective activator of cPKC, caused a similar reduction in hCAT-3 activity as PMA, and this effect could also be attenuated by Ro 31-8220 (1 μM). In contrast, PIP (phosphatidylinositol-3,4,5-

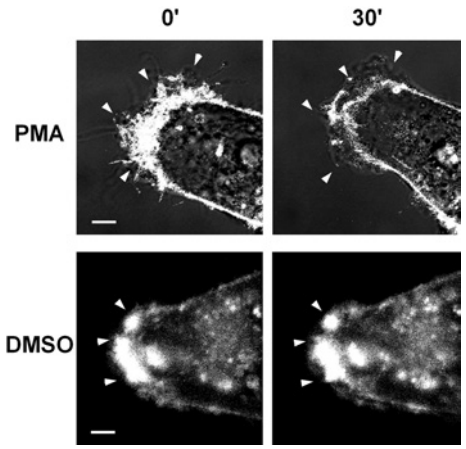

Figure 3 PMA-induced reduction in the cell-surface expression of hCAT-3.EGFP in U373MG cells

U373MG glioblastoma cells stably transfected with hCAT-3.EGFP and grown on coverglass-bottom chamber slides were treated with 0.1 % DMSO or with 100 nM PMA (in 0.1 % DMSO) for the times indicated and analysed by confocal microscopy. The Figure shows for each treatment succeeding confocal micrographs of the same cell collected under identical scanning parameters. The fluorescent and differential interference contrast micrographs were overlaid to visualize the ruffled borders (arrowheads) where hCAT-3.EGFP expression was strongest in control cells. Scale bars represent 10 μm.

trisphosphate-dipalmitoyl; 5 μM), an activator of nPKC isoforms had no effect on hCAT-3-mediated transport. These results support the notion that nPKCs are not involved in the down-regulation of hCAT-3. Consequently, it is most likely a cPKC isoform that triggers the reduction in hCAT-3 activity. This is again similar to the situation of hCAT-1 in endothelial cells, that seems to be down-regulated by a cPKC isoform, probably PKCα [4]. In U373MG cells, the cPKC isoforms α and γ have been detected and shown to be activated by PMA [21]. In these cells, PMA increases also the expression and activity of the aPKC isoform ζ, most probably through lipid second messengers. However, this requires a prolonged PMA treatment and is not inhibited by BIM I. Therefore an involvement of PKCζ in the down-regulation of hCAT-3 after a 30 min PMA treatment is very unlikely. Further studies are needed to determine which of the two cPKC isoforms α or γ is responsible for decreasing hCAT-3 activity.

PKC-mediated reduction of hCAT-3 expression in the plasma membrane of human glioblastoma cells

hCAT-3.EGFP stably expressed in several independent U373MG glioblastoma cell clones was localized in the plasma membrane as well as in intracellular vesicles, as evidenced by confocal microscopy. In the plasma membrane, a distinct fluorescence pattern was detected with the strongest fluorescence intensity always observed in the ruffled borders (Figure 3). A 15–30 min treatment of the cells with 100 nM PMA led to a marked down-regulation of hCAT-3.EGFP expressed in the plasma membrane. In contrast, the subcellular distribution did not change upon treatment of cells with DMSO alone. To quantify the cell-surface expression of hCAT-3, cell-surface proteins were biotinylated and separated from internal proteins using avidin-coated beads. In Western blots of these cell-surface protein fractions, no signal for the cytosolic β-tubulin was detected, confirming the efficiency of the separation method (Figure 4A, lower panel). After PMA treatment, hCAT-3.EGFP was reduced in the cell-surface protein fraction (65 ± 4.4 % of control), but not in total cell lysates (Figure 4). The PMA effect on the cell-surface expression was

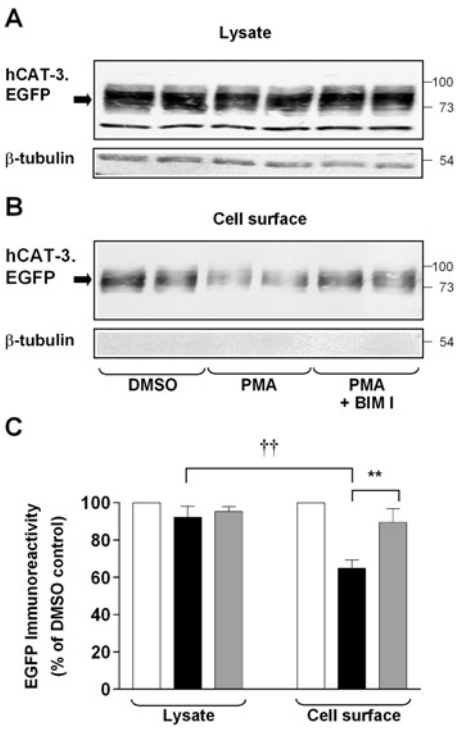

Figure 4 Quantification of the PMA-induced internalization of hCAT-3.EGFP expressed in human U373MG cells

Confluent U373MG glioblastoma cells stably transfected with hCAT-3.EGFP and grown in 10 cm dishes were first treated for 5 min with 1 μM BIM I (lanes with BIM I) or 0.1 % DMSO alone (all other lanes) and then for further 30 min as indicated with 0.15 % DMSO alone, 100 nM PMA or 100 nM PMA plus 0.5 μM BIM I. Cell-surface proteins were then biotinylated and separated from non-biotinylated proteins as specified in the Experimental section. (**A**, **B**) Western blot with total cell lysates (15 μg/lane) (**A**) and cell-surface proteins (**B**) separated by SDS/PAGE (10 % gel) and probed with an anti-GFP antibody (upper panels) and subsequently with an anti-tubulin antibody as control (lower panels). (**C**) Protein expression was quantified by densitometry of six independent experiments (means $\pm$ S.E.M.; white bars, DMSO control; black bars, PMA; grey bars, PMA plus BIM I). Statistical analysis was performed using ANOVA with the Bonferroni post hoc test (**$P < 0.01$ between samples treated with PMA only and with PMA plus BIM I; ††$P < 0.01$ between PMA-treated total cell lysates and cell-surface proteins).

attenuated in the presence of the PKC inhibitor BIM I (90 $\pm$ 7 % of control). These results suggest that, as for hCAT-1, PKC activation leads to a reduced cell-surface expression of hCAT-3 without reducing the overall hCAT-3 protein expression.

Our previous work demonstrated that PKC activation does not lead to an enhanced degradation of hCAT-1 and suggested that the PKC-induced internalization is reversible. PKC thus seems to influence exclusively the trafficking of hCAT-1 (results not shown). In addition, PKC does not phosphorylate hCAT-1 either directly or indirectly, indicating its action on intermediate proteins that interact with hCAT-1. The similar response of hCAT-3 to PKC activation suggests that its subcellular distribution is regulated in a similar way to hCAT-1. An indirect regulation of transporter localization and/or activity by PKC has also been demonstrated for a number of other unrelated transporters (for example, see [22,23]). The effect of PKC on these transporters seems to be dependent on intermediate or associated proteins. For example, the PKC-dependent decrease in cell-surface expression of the GABA (γ-aminobutyric acid) transporter GAT1 (GABA transporter 1) is dependent on SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins, including syntaxin 1 [24]. The PKCα-binding protein PICK1 (protein interacting with C-kinase 1) associates with the

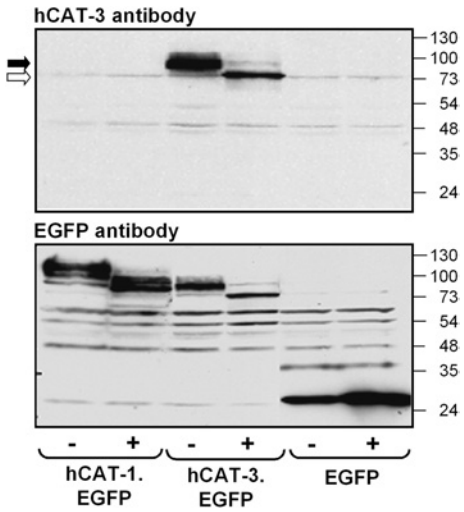

Figure 5 Specificity of the affinity-purified antibody against hCAT-3

Western blots were performed with lysates from U373 cells stably expressing hCAT-1.EGFP or hCAT-3.EGFP fusion proteins or EGFP alone. In lanes marked '+', lysates were treated with PNGase F prior to SDS/PAGE. In lanes marked '—', lysates were untreated. Protein, 20 μg per lane, was separated by SDS/PAGE (10 % gel), blotted and the membrane was probed with the affinity-purified anti-hCAT-3 antibody (upper panel) or with an anti-EGFP antibody (lower panel). Black and white arrows in the upper panel indicate the glycosylated and deglycosylated hCAT-3.EGFP protein respectively.

dopamine transporter DAT and might be responsible for PKC-induced internalization of this transporter [25]. The inhibitory action of PKC on the 5-hydroxytryptamine transporter SERT (serotonin transporter) may be mediated by disruption of its association with the phosphatase PP2A (protein phosphatase 2A) [26]. It thus seems very likely that associated proteins also mediate PKC regulation of hCAT-3. Differences in interacting proteins might also explain the opposite effect of PKC activation on CAT-mediated transport in different cell types. Such opposite effects have also been observed for other transporters. For example, extracellular PMA induces internalization and decreased activity of GAT1 in mammalian cells and *X. laevis* oocytes, while intracellular administration of PMA in oocytes increases GAT1 activity [24,27]. Similarly, PMA induces a decrease in the glutamate transporter EAAC1 in oocytes, but increases its activity in mammalian cells [28,29].

PKC-mediated reduction of endogenous hCAT-3 in the plasma membrane of human teratocarcinoma cells

As our studies were performed on hCAT-3 overexpressed exogenously in *X. laevis* oocytes or human cells, we aimed at confirming that PKC activation has a similar effect on endogenous hCAT-3. In a screen of various human cell lines, NT2 teratocarcinoma cells were the only cells exhibiting significant hCAT-3 expression (results not shown) and were therefore chosen for our analyses. For detection of the endogenous hCAT-3, an isoform-specific antibody was made against the C-terminus of hCAT-3. This antibody recognized bands of approx. 80 and 100 kDa respectively in PNGase F-treated and untreated lysates from cells overexpressing hCAT-3.EGFP (Figure 5). In contrast, no signal was seen in lysates from control cells expressing hCAT-1.EGFP or EGFP alone, demonstrating that the antibody recognizes specifically hCAT-3.

The expression of endogenous hCAT-3 in the plasma membrane was significantly reduced upon a 2 h PMA treatment of NT2 cells, as shown by Western blots performed with the biotinylated

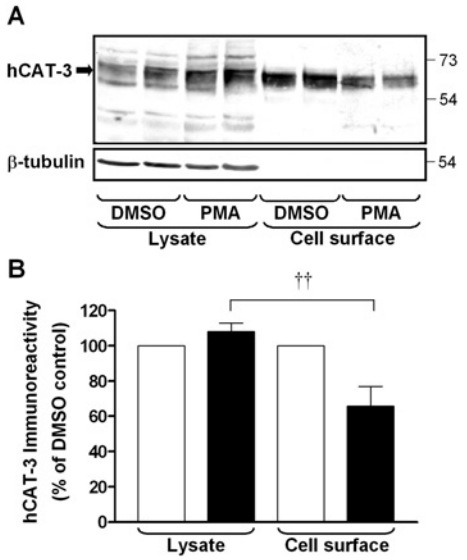

Figure 6 PMA-induced internalization of endogenous hCAT-3 expressed in human NT2 teratocarcinoma cells

Confluent NT2 cells grown in 10 cm dishes were first treated for 30 min as indicated with 0.1 % DMSO alone or in addition with 100 nM PMA. Cell-surface proteins were then biotinylated and separated from non-biotinylated proteins as specified in the Experimental section. (**A**) Western blot with total cell lysates (100 μg/lane) and cell-surface proteins separated by SDS/PAGE (10 % gel) and probed with an anti-hCAT-3-antibody (upper panel) and subsequently with an anti-tubulin antibody as control (lower panel). (**B**) Protein expression was quantified by densitometry of four independent experiments (means $\pm$ S.E.M.; white bars, DMSO control; black bars, PMA). Statistical analysis was performed using ANOVA with the Bonferroni post hoc test (††$P < 0.01$ between PMA-treated total cell lysates and cell-surface proteins).

membrane fractions (Figure 6). In contrast, PMA had no effect on the overall content of hCAT-3 measured in unfractionated cell lysates. This is consistent with the results for the overexpressed transporter described above. NT2 cells tolerated the PMA treatment only when grown on poly(L-lysine)-coated plates. Under these conditions, a 30 min PMA treatment altered neither L-arginine transport nor the cell-surface expression of hCAT-3 in these cells (results not shown). Even when grown on coated plates, a 2 h PMA treatment reduced the adhesiveness of NT2 cells such that we could not perform transport studies under these conditions. It is not clear why the reduction in cell-surface expression of hCAT-3 took longer in NT2 than in U373 cells. This may reflect differences between cell lines or be due to the coating of the culture plates. As observed for hCAT-3, the cell-surface expression of hCAT-1 in NT2 cells was unchanged after 30 min PMA treatment and reduced after 2 h (results not shown). This demonstrates that PKC activation regulates both transporters in a similar way.

Biotinylation experiments revealed a similar percentage of hCAT-3 in the membrane fraction of both cells expressing the transporter endogenously and cells overexpressing an EGFP fusion protein (Supplementary Figures A and B at http://www.BiochemJ.org/bj/395/bj3950117add.htm). Also, the subcellular distribution of hCAT-3.EGFP seemed to be similar to hCAT-1.EGFP (Supplementary Figures B and C at http://www.BiochemJ.org/bj/395/bj3950117add.htm). The relatively small percentage of transporter protein detected in the plasma membrane fraction (between 12 and 21 %) is in line with our observation on fluorescent micrographs showing a significant portion of each transporter located in intracellular membranes (Figure 3). However, we cannot exactly quantify the percentage of hCAT-3 in the plasma membrane as the efficacy of the biotinylation reaction is not known.

Conclusions

Our study shows that PKC activation induces a decrease in cell-surface expression of hCAT-3 expressed either endogenously or exogenously. The reduced cell-surface expression observed for fusion proteins between hCAT-3 and EGFP overexpressed in *X. laevis* oocytes and in U373MG human glioblastoma cells was accompanied by a similar reduction in hCAT-3-mediated transport. These results indicate that the PKC-induced transport inhibition is the result of transporter internalization rather than modifications in hCAT-3 catalytic activity.

The profile of PKC inhibitors and activators indicates that a cPKC isoform is responsible for reducing the cell-surface expression of hCAT-3. This is similar to hCAT-1 that is also down-regulated by cPKC [4]. cPKCα and cPKCγ are expressed in both cell types investigated and are thus the candidates for regulating the subcellular distribution of hCAT-1 and hCAT-3. Both isoforms are also expressed in macrophages where an up-regulation of system y^+-mediated arginine transport in response to PKC activation has been reported [30]. It thus seems unlikely that the opposite effects of PKC activation on system y^+-mediated transport observed in the different cell types are due to differences in PKC isoforms expressed in the respective cells. It seems instead more likely that the opposite effects are due to differences in intermediate proteins conveying the respective PKC effect. The involvement of such mediators is also sustained by the finding that PKC activation does not lead to an altered phosphorylation of hCAT-1 [5]. The identification of proteins that interact with the CAT proteins will be crucial to understand how the cell-surface expression of these proteins is regulated.

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