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The transport activity of the human cationic amino acid transporter hCAT-1 is downregulated by activation of protein kinase C

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1 The human cationic amino acid transporter hCAT-1 contains several consensus sequences for phosphorylation by protein kinase C (PKC). This study investigates the effect of PKC activation on hCAT-1-mediated transport.

2 When expressed in *Xenopus laevis* oocytes, hCAT-1-mediated L-arginine transport was reduced to 44±3% after a 30 min treatment of the oocytes with 100 nM phorbol-12-myristate-13-acetate (PMA). 4α-phorbol-12,13-didecanoate (4α-PDD, 100 nM) had no effect.

3 In EA.hy926 endothelial cells, maximal inhibition of hCAT-1-mediated L-arginine transport (to 3–11% of control) was observed after treatment of the cells with 100 nM PMA for 4 h. A 20–30 h exposure of the cells to 100 nM PMA led to the recovery of the L-arginine uptake rate that was now resistant to a second application of PMA. Phorbol-12,13-dibutyrate had similar effects as PMA, whereas 4α-PDD had no effect. One μM bisindolylmaleimide I reduced the PMA effect significantly.

4 Interestingly, a 4 h treatment with 100 nM PMA increased the expression of hCAT-1 mRNA 3–5 fold. hCAT-1 protein levels were unchanged for up to 4 h after PMA treatment and then increased slightly between 8–28 h.

5 It is concluded that PMA downregulates the intrinsic activity of hCAT-1 by a pathway involving protein kinase C.

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Abbreviations: BIM I, bisindolylmaleimide I; DMEM, Dulbecco's modified Eagle's medium; DMSO; dimethyl sulphoxide; hCAT; human cationic amino acid transporter; HUVEC, human umbilical vein endothelial cell; LPC, lysophosphatidylcholine; nt, nucleotides; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PDBu, phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulphate

Introduction

The cationic amino acid transporter-1 (CAT-1) originally identified in murine cells, most likely represents the major carrier for cationic amino acids in most mammalian cells (for review see Closs, 1996; Closs & Gräf, 1999a; Deves & Boyd, 1998; Macleod & Kakuda, 1996). CAT-1 is well conserved amongst mammalian species. The deduced amino acid sequences of mouse (Albritton *et al*., 1989), rat (Puppi & Henning, 1995; Wu *et al*., 1994) and human (Albritton *et al*., 1993; Yoshimoto *et al*., 1991) CAT-1 are 95.8% (mouse and rat) and 86.6% (mouse and human) identical. Three conserved consensus sequences for phosphorylation by protein kinase C (PKC) are located in the fifth and sixth putative intracellular loops according to the model by Albritton and co-workers that suggests 14 membrane-spanning domains (Albritton *et al*., 1989). Therefore, PKC phosphorylation might influence the transport activity or subcellular distribution of CAT-1. The transport properties of mouse and human CAT-1 are almost identical as determined in transport studies on *Xenopus laevis* oocytes, where each carrier can be expressed to a high level (Closs *et al*., 1997; Kim *et al*., 1991; Wang *et al*., 1991). The transport characteristics of CAT-1 are consistent with the so-called system y^+ (for review see Deves & Boyd, 1998). With the exception of the liver, CAT-1 is ubiquitously expressed in rodent and human tissues and cell lines (Closs *et al*., 1993; Kim & Cunningham, 1993; Wu *et al*., 1994; Yoshimoto *et al*., 1992). A specific role of CAT-1 for supplying the substrate L-arginine to the endothelial nitric oxide synthase (NOS III) has been proposed, based on immunostainings of porcine pulmonary artery endothelial cells that indicate a co-localization of CAT-1, NOS III and caveolin in endothelial caveoli (McDonald *et al*., 1997). Further support for the importance of CAT-1-mediated L-arginine transport for endothelial NO synthesis comes from a more recent study in bovine aortic endothelial cells (Kikuta *et al*., 1998). Treatment of these cells with lysophosphatidylcholine (LPC) inhibits both, the high affinity component of the L-arginine transport (presumably CAT-1) and ADP-induced NO release. In contrast, our previous results show that the NOS III activity in human endothelial EA.hy926 cells is largely independent of extracellular L-arginine indicating that the substrate transport is not rate limiting for NO synthesis in these cells (Closs *et al*., 2000).

PKC-activation seems to have differential effects on CAT-1 expression. In B-lymphocytes, treatment with the PKC

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activating phorbol ester, phorbol-12-myristate-13-acetate (PMA) or with the Ca^{2+} ionophore A23187, leads to a massive increase in CAT-1 mRNA levels concomitant with an induction of cell proliferation (Yoshimoto *et al*., 1992). In contrast, in the promyelocytic cell line HL60, PMA downregulates CAT-1 mRNA expression while it induces macrophage differentiation and cessation of proliferation.

Studies investigating L-arginine transport in different cell types also show differential effects of PKC activation. In SK-Hep1 human hepatoma cells, PMA treatment leads to a decrease in the rate of L-arginine transport (Bode *et al*., 1998). In contrast, in human umbilical vein endothelial cells (HUVEC) (Pan *et al*., 1995b), human Caco intestinal epithelial cells (Pan *et al*., 1995a), and in rat, rabbit, and mouse peritoneal macrophages (Hortelano *et al*., 1993; Racke *et al*., 1998), PKC activation causes an increase in the L-arginine transport rate. For the PMA-induced upregulation of the L-arginine transport, protein synthesis is required in HUVEC and Caco cells, but not in macrophages, suggesting that the mechanism of transport induction differs in different cell types.

In this study, we demonstrate that PKC activation leads to a decrease in the activity of hCAT-1 expressed in *Xenopus laevis* oocytes. In addition, we investigate the effect of PKC activation on hCAT-1 activity in the human endothelial cell line EA.hy926. Based on mRNA expression and transport studies, CAT-1 seems to be the only carrier for cationic amino acids in these cells (Closs *et al*., 2000). Exposure to PMA leads to a temporary activation (2–6 h) and subsequent degradation of PKCα and ε in these cells (Li *et al*., 1998). Our present data show that PKC activation also temporarily decreases hCAT-1 activity in EA.hy926 cells without reducing hCAT-1 protein expression.

Methods

Expression of cRNAs in Xenopus laevis *oocytes*

A construct containing the complete coding sequence of hCAT-1 (pSPhCAT-1AB1) in pSP64T has been described previously (Closs *et al*., 1997). The plasmid was linearized with *Sal*I and cRNA was prepared by *in vitro* transcription from the SP6 promoter of pSP64T (mMessage mMachine *in vitro* transcription kit, Ambion, AMS Biotechnology Europe, Wiesbaden, Germany). Twenty-five ng cRNA (in 25 nl H_2O) were injected into each *Xenopus laevis* oocyte (Dumont stage VI-VII). Oocytes injected with 25 nl water were used as controls.

Measurement of L-arginine uptake in Xenopus laevis *oocytes*

L-arginine uptake was determined 3 days after injection of cRNA as previously described (Kim *et al*., 1991). Briefly, oocytes were equilibrated for 2 h at 20°C in 'uptake solution' (mM: NaCl 100, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, Tris 5, pH 7.5) containing 100 μM unlabelled L-arginine. After the incubation, oocytes were first transferred to the same solution containing 100 nM PMA, 100 nM 4α-phorbol-12,13-didecanoate (4α-PDD), each in 0.1% dimethyl sulphoxide (DMSO), or 0.1% DMSO alone (30 min, 20°C) and then to 'uptake solution' containing 100 μM [2,3-^{3}H]-L-arginine (5 μCi ml^{-1}, Dupont NEN, Bad Homburg, Germany, 15 min, 20°C). The oocytes were washed five times in ice cold 'uptake solution' and solubilized individually in 1% sodium dodecyl sulphate (SDS). The incorporated radioactivity was determined in a liquid scintillation counter.

Cell culture

The human endothelial cell line EA.hy926 was a gift from C-J.S. Edgell (University of North Carolina at Chapel Hill, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 4 mM glutamine and 10% foetal bovine serum. Cells were regularly tested for mycoplasma infection using 4′,6 diamindine.2′-phenylindole dihydrochloride (DAPI, Roche Molecular Biochemicals, Mannheim, Germany). No contamination could be detected.

Measurement of L-arginine transport in EA.hy926 cells

Cells grown to confluence in 96-well plates (diameter 0.7 cm) were washed twice with Locke's solution (in mM: NaCl 154, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, $NaHCO_3$ 3.6, glucose 5.6) containing a defined concentration of L-arginine and then incubated at 37°C for the times indicated in 100 μl well^{-1} of the same solution containing PMA or other compounds in the concentration given in the figure legend. After the preincubation the Locke's solution was exchanged with the same solution containing L-[^{3}H]-arginine (5 μCi ml^{-1}), but no PMA, and the cells were incubated for 60 s at 37°C. The cells were then immediately transferred on ice, washed three times with ice cold Locke's solution and lysed in 0.5 M NaOH (25 μl well^{-1}, 30 min at 4°C). After neutralization of the lysates with 25 μl 0.5 M HCl and 50 μl buffer A (50 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA) the protein content of each sample was determined using the Bradford reaction (BioRad, Munich, Germany). The radioactivity in the samples was measured by liquid scintillation counting. The background radioactivity derived from L-arginine bound to the cells (determined by addition of Locke's solution containing L-[^{3}H]-arginine (5 μCi ml^{-1}) followed by immediate washing steps) was subtracted from all values.

Ribonuclease protection analyses

A probe for hCAT-1 was obtained by subcloning the *Nco*I/*Asp* 718 fragment from phCAT-1 AB1 (Closs *et al*., 1997) into pBluescriptKS$^+$ (Stratagene, Heidelberg, Germany) resulting in phCAT-1/riboII. A plasmid containing a cDNA fragment of the human β-actin cDNA (pCR_β-actin_hu, 353 nt) has previously been generated (Kleinert *et al*., 1996). The pCR_β-actin_hu was restricted with *Bst*EII and *Hind*III, blunted and religated resulting in pCR_β-actin_hu_Δ*Bst*EII_*Hind*III that contains only 108 nt of the human β-actin cDNA. To generate radiolabelled antisense RNA probes, phCAT-1/riboII was linearized with *Xba*I, and pCRβ-actin_hu_Δ*Bst*EII_*Hind*III with *Asp* 718. *In vitro* transcription was performed as previously described (Closs & Mann, 1999b) resulting in α-^{32}P-labelled riboprobes of 252 nt for hCAT-1, and 222 nt for hβ-actin. Total RNA was isolated from endothelial cells using the method of Chomczynski &

Sacchi (1987). Ribonuclease protection analyses were performed with 20 μg RNA per sample as described (Closs & Mann, 1999b).

Generation of an immune plasma specific for hCAT-1

A cDNA fragment coding for the 49 carboxyterminal amino acids of hCAT-1 was cloned in frame into pATH-1, 3′ to the coding region of tryptophane E (trpE) (Koerner *et al*., 1991). The resulting plasmid was transfected into *E. coli* XL-1 blue (Stratagene, Heidelberg, Germany). Expression of the trpE/hCAT-1 fusion protein was induced by growth in tryptophane-free M9 medium containing 10 μg ml^{-1} 3,β-indoleacrylic acid (Sigma, Deisenhofen, Germany) (4 h, 37°C). Bacteria were lysed by sonication in HEMGN buffer (mM: KCl 100, HEPES 25, pH 7.6; EDTA 0.1, $MgCl_2$ 12, 10% glycerol; 0.1% Nonidet P-40) containing 1 mM DTT; 2 μg ml^{-1} aprotinin; 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin; 0.1 mM phenymethylsulphonyl fluoride; 0.2 mM $NaHSO_3$ and 0.5 mg ml^{-1} lysozyme. The lysates were spun at 26,000 × *g* and the supernatants (about 15 mg protein) separated by 12.5% SDS polyacrylamide gel electrophoresis (PAGE). After Coomassie Blue staining the gel portion containing the fusion protein was cut out and homogenized in 1 ml H_2O g^{-1} gel using a 26 gauge needle. Six-week-old rabbits were immunized with 300 μl homogenate (250 μg fusion protein) and an equal volume complete Freund's adjuvans (Life Technologies, Eggenstein, Germany). The immune plasma was collected after boosting the rabbits three times (every 3 weeks) with 250 μg fusion protein (in 300 μl) and 1 volume of incomplete Freund's adjuvans.

Affinity purification of immune plasma to hCAT-1

A cDNA fragment coding for the 25 carboxyterminal amino acids of hCAT-1 was cloned in frame into pGEX-2T, 3′ to the coding region of gluthathione S transferase (GST) (Smith & Johnson, 1988). The resulting plasmid was transfected into *E. coli* XL-1 blue (Stratagene, Heidelberg, Germany). Expression of the GST/hCAT-1 fusion protein was induced by growth in LB medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside (Roche Molecular Biochemicals, Mannheim, Germany, 5 h, 37°C). Bacteria were lysed on ice by sonication in phosphate buffered saline (PBS, mM: KCl 2.7, NaCl 140, KH_2PO_4 1.8, Na_2HPO_4 10). After addition of 0.1% Triton-X-100 the lysates were spun at 7500 × *g* and the fusion protein in the supernatants purified using S-glutathione sepharose (Simons & Vander, 1981). Two ml immune plasma was heat inactivated (30 min, 56°C), diluted 1 + 1 with PBS and applied to a Poly-Prep chromatography column (BioRad, Munich, Germany) containing 8 mg GST/hCAT-1 fusion protein coupled to 4 ml Affigel 10 (BioRad, Munich, Germany, 4°C, 15 h). After washing the column with 12 ml PBS, antibodies were eluted with 0.1 M glycine/HCl pH 2.5, 1 M NaCl, neutralized with 1/10 volume 1 M Tris/HCl pH 8 and dialysed against PBS (4°C, 15 h).

Protein lysates and Western blot

Oocytes were lysed by vortexing in RIPA buffer (1% deoxycholate; 1% Triton-X-100; 0.1% SDS; 150 mM NaCl; 2 mM $MgCl_2$; 10 mM Tris/HCl pH 7.2; 1 mM phenymethylsulphonyl fluoride) 3 days after injection with hCAT-1 or hCAT-2A cRNA or with water (10 oocytes 50 μl^{-1} buffer). After determining the protein concentration (using the Bradford reaction) the lysates were treated with N-glycosidase F (Roche Molecular Biochemicals, Mannheim, Germany, 10 U 100 μg^{-1} for 1 h at 37°C). An equal volume RIPA buffer containing 8 M urea was then added and the samples spun at 14,000 × *g*.

EA.hy926 cells grown to confluence in culture plates (diameter: 10 cm) were washed three times with PBS, scraped from the plates and pelleted at 180 g. Cells were then lysed with two volumes of NP-40 buffer (10 mM Tris/HCl pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$; 0.5% Nonidet P-40; 1 mM phenymethylsulphonyl fluoride; 0.3 μg/ml aprotinin; 1 μg/ml leupeptin; 1 mg/ml pepstatin) and the nuclei pelleted at 720 g. The protein concentration of the supernatants was determined using the Bradford reaction and the lysates were treated with N-glycosidase F where indicated (10 u 100 μg^{-1} for 1 h at 37°C).

Lysates (40 μg protein) were separated in 10% SDS polyacrylamide gel electrophoresis (PAGE) and then blotted to nitrocellulose membranes (Protran83, Schleicher and Schuell, Dassel, Germany). Staining for hCAT-1 protein was achieved by sequential incubations in: Blotto (50 mM Tris/HCl pH 8; 2 mM $CaCl_2$; 0.01% antifoam A (Sigma, Deisenhofen, Germany); 0.05% Tween 20; 0.02% NaN_3, 5% nonfat dry milk) containing 10% goat serum (1 h, room temperature), a 1 : 100 dilution of the anti-hCAT-1 immune plasma or the affinity-purified antibodies in PBS containing 1% bovine serum albumin, 0.1% Tween 20 and 0.2% NaN_3 (over night, 4°C), three times 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) (2 h, room temperature); three times TBS (10 mM Tris/HCl pH 8; 150 mM NaCl; 0.05% Tween 20) and chemoluminescence reagent (Renaissance, Dupont NEN, Bad Homburg, Germany) (1 min). The signals on the membranes were then immediately quantified using a chemiluminescence detection system (BioRad, Munich, Germany). For standardization, membranes were stripped (in 62.5 mM Tris/HCl pH 6.8; 2% SDS, 100 mM β-mercaptoethanol, 30 min, 50°C) and stained with a monoclonal antibody to β-tubulin (1 : 1000, Sigma Deisenhofen, Germany) and a peroxidase-conjugated secondary goat anti-mouse IgG antibody (1 : 3000, Sigma, Deisenhofen, Germany).

Statistics

Results were compared for statistical differences using analysis of variance with the Bonferroni post test. *P*-values <0.05, <0.01, <0.001 were considered significant and marked by one, two, and three asterisks, respectively. Absence of significance was marked by ns.

Results

A potential effect of PKC activation on hCAT-1-mediated transport was initially studied in *Xenopus laevis* oocytes expressing hCAT-1. Three days after injection of hCAT-1 cRNA (25 ng 25 nl^{-1}), oocytes were treated for 30 min with

100 nM PMA, 100 nM 4α-PDD (both in 0.1% DMSO) or 0.1% DMSO only and the uptake of 100 μM L-[^{3}H]-arginine was measured. In oocytes treated with the PKC activating phorbol ester PMA, hCAT-1-mediated transport was reduced to 44±3% of control (Figure 1). In contrast, the inactive phorbol ester, 4α-PDD, or DMSO used as the solvent for the phorbol, esters had no effect.

We then studied the effect of PKC activation on L-arginine transport in the human endothelial cell line EA.hy926 that we have previously shown to express hCAT-1 only (Closs *et al*., 2000). A 20 min incubation of EA.hy926 cells in 100 nM PMA reduced the maximal velocity (V_{max}) of the L-arginine transport from 10.3 to 4.6 pmol μg^{-1} protein min^{-1} (Figure 2). Half maximal transport rates were reached at an L-arginine concentration (K_M) of about 90 μM in both PMA-treated cells and controls. The PMA-induced inhibition of the L-arginine transport in EA.hy926 cells was time-dependent. Figure 3A shows a significant reduction to 57±6% of control as soon as 5 min after addition of 100 nM PMA. Maximal inhibition to 5–11% of control occurred after a 1–4 h treatment with 100 nM PMA (Figure 3A,B). In cells exposed 20–28 h to 100 nM PMA, L-arginine transport was restored to 49±5% compared to the transport before the PMA treatment (Figure 3B) and 73±3% compared to cells treated for 28 h with 0.1% DMSO only (Figure 3C). Application of fresh PMA (100 nM) to cells preincubated for 28 h with the same concentration of PMA had no effect on L-arginine transport (Figure 3C). The inhibitory effect of PMA on the L-arginine transport in EA.hy926 cells was concentration-dependent (Figure 4). When exposing the cells to PMA for 4 h, half maximal inhibition was reached at a PMA concentration of 20 nM. To investigate if the PMA effect was mediated by an activation of PKC, we measured L-arginine transport in the presence of other phorbol esters and

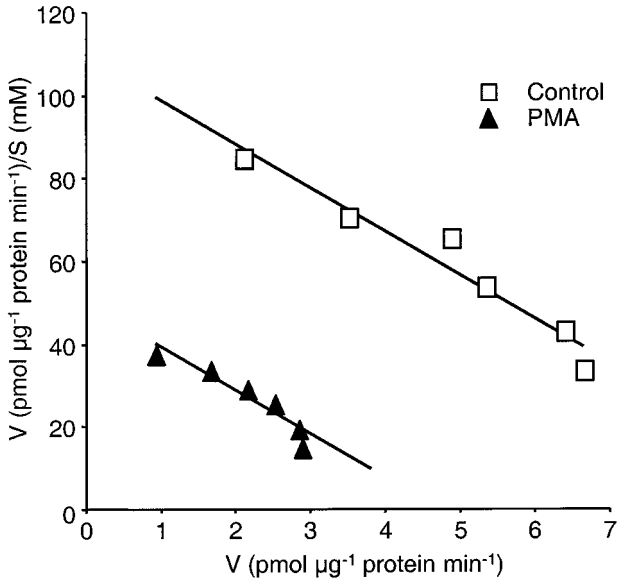

Figure 2 Reduced maximal velocity of the L-arginine transporter in EA.hy926 endothelial cells after treatment with PMA (Eadie-Hofstee plots of L-[^{3}H]-arginine uptake). Confluent cells were equilibrated for 2 h with a defined concentration of unlabelled L-arginine (25, 50, 75, 100, 150 or 200 μM) in Locke's solution. The supernatants were then exchanged for Locke's solution containing the same concentrations of cold L-arginine and either 100 nM PMA (in 0.1% DMSO) or only 0.1% DMSO (control), and the cells incubated for further 20 min. L-[^{3}H]-arginine (5 $\mu Ci\ ml^{-1}$) in the same concentration as used for the equilibration was then added and the cells were further incubated for 60 s at 37°C. The cells were then washed extensively, lysed and radioactivity and protein content of the lysates was determined ($n=6$). No difference was found between untreated cells and cells treated with 0.1% DMSO only (data not shown).

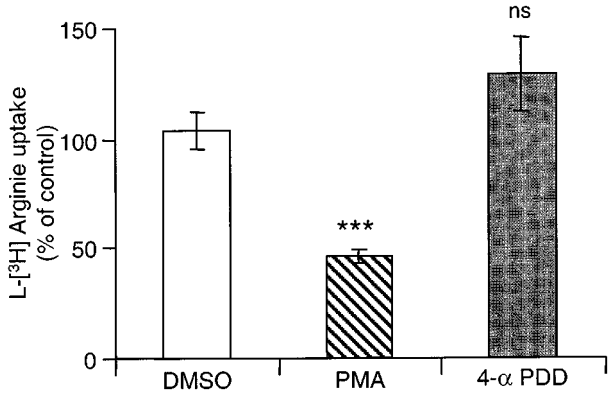

Figure 1 Downregulation of hCAT-1-mediated L-arginine transport in *Xenopus laevis* oocytes by PMA (L-[^{3}H]-arginine uptake studies). Three days after injection of hCAT-1 cRNA, oocytes were first preincubated in uptake buffer containing 100 μM L-arginine for 2 h and then for 30 min in the same buffer containing 0.1% DMSO, 100 nM PMA (in 0.1% DMSO) or 100 nM 4α-PDD (in 0.1% DMSO). The oocytes were then transferred to the 'uptake solution' containing 100 μM L-[^{3}H]-arginine and incubated for 15 min. The cells were then washed extensively, lysed and the radioactivity determined. Values obtained with water-injected oocytes (4–10 pmol $oocyte^{-1}\ h^{-1}$) were subtracted. Data are expressed as per cent of the transport rate of untreated oocytes (100–300 pmol $oocyte^{-1}\ h^{-1}$), (means±s.e.mean, $n=12–16$). Asterisks indicate differences from DMSO-treated samples, ***: $P<0.001$, ns: not significant.

the PKC inhibitor bisindolylmaleimide I (BIM I, Figure 5). One μM BIM I alone had no effect on the L-arginine transport in EA.hy926 cells (Figure 5A). However, BIM I at the same concentration significantly reduced the inhibition of L-arginine transport induced by 100 nM PMA, from 97±1% to 54±2%. Incubation of the EA.hy cells in 100 nM of the inactive phorbol ester 4α-PDD did not reduce the transport activity for L-arginine (Figure 5B). In contrast, exposure to 100 nM phorbol-12,13-dibutyrate (PDBu), another PKC activator, for 4 h led to an inhibition of the L-arginine transport to 22±3% of controls.

We next investigated whether the expression of hCAT-1 in EA.hy926 cells was changed by a treatment with PMA. Surprisingly, the expression of hCAT-1 mRNA was increased by PMA as determined by ribonuclease protection analyses (Figure 6). Quantitative analyses of the hCAT-1 signal (normalized to β-actin) demonstrated a 3–5 fold increase in the hCAT-1 mRNA levels 4 h after addition of 100 nM PMA. After 30 h, the hCAT-1 mRNA levels were still about 2 fold higher than in controls. In cells treated for the same time periods with 0.1% DMSO only, no change in hCAT-1 mRNA expression was observed (data not shown). To investigate the expression of the hCAT-1 protein, antibodies were raised against the C-terminus of hCAT-1 using fusion proteins between trpE and the C-terminal 49 amino acids of hCAT-1. The immune plasma of one rabbit (αhCAT-1/Kh5) specifically recognized a band in Western blots of N-

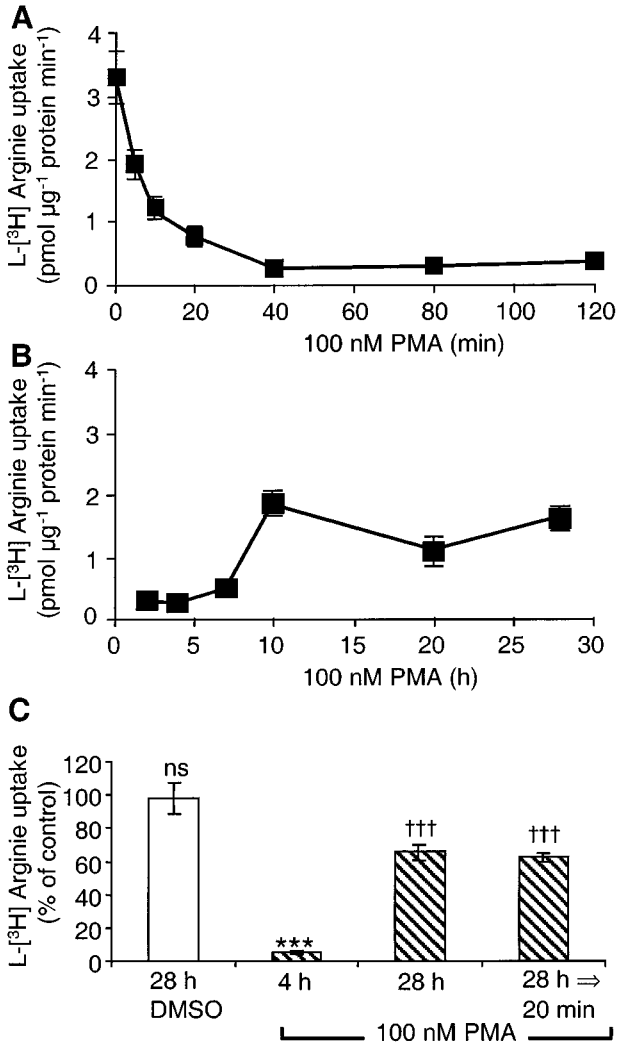

Figure 3 Time dependence of the PMA effect on L-arginine transport in EA.hy926 cells (L-[^{3}H]-arginine uptake studies). Confluent cells grown in 96 well plates were incubated with Locke's solution supplemented with 100 μM unlabelled L-arginine and then with the same solution containing 100 nM PMA (final DMSO concentration: 0.1%) (A) for 5, 10, 20, 40, 80 or 120 min; (B) for 2, 4, 7, 10, 20 or 28 h; (C) for 4 h, 28 h or 28 h and a second application of 100 nM PMA for 20 min, DMSO: cells incubated for 28 h with only Locke's solution and 0.1% DMSO. Locke's solution containing 100 μM L-[^{3}H]-arginine (5 μCi ml^{-1}) was then added and the cells were further incubated for 60 s at 37°C. The cells were then washed extensively, lysed and radioactivity and protein content of the lysates were determined. A+B: Data represent means±s.e.mean, $n=6$. (C) Data are expressed as per cent of the transport rate of untreated cells (2.68±0.19 pmol μg^{-1} protein min^{-1}, $n=12$). Asterisks indicate differences from DMSO-treated samples, ***: $P<0.001$, crosses indicate differences from samples treated with PMA for 4 h, †††: $P<0.001$, ns: not significant.

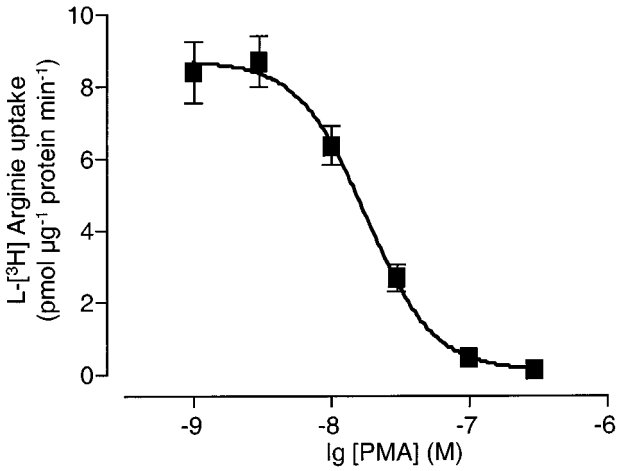

Figure 4 Concentration dependence of the PMA effect on L-arginine transport in EA.hy926 cells (L-[^{3}H]-arginine uptake studies). Confluent cells were incubated for 4 h in Locke's solution supplemented with 100 μM unlabelled L-arginine and 1, 3, 10, 30, 100 or 300 nM PMA (final DMSO concentration: 0.3%). Locke's solution containing 100 μM L-[^{3}H]-arginine (5 μCi ml^{-1}) was then added and the cells were incubated for another 60 s at 37°C. Cells were then washed extensively, lysed and radioactivity and protein content of the lysates was determined (means±s.e.mean, $n=6$). No difference in L-arginine transport was found in cells treated with 0.3% DMSO only and untreated cells (data not shown).

glycosidase F-treated lysates from hCAT-1-expressing oocytes, but not from hCAT-2A-expressing oocytes or from water-injected controls (Figure 7A). The identified band migrated at about 67 kDa, the predicted molecular mass of the deglycosylated hCAT-1, calculated from the amino acid composition. In Western blots, using affinity purified αhCAT-1/Kh5, an even stronger hCAT-1-specific signal could be detected (Figure 7B). The affinity-purified αhCAT-1/Kh5 recognized a band in Western blots with N-glycosidase F-treated lysates from EA.hy926 cells that co-migrated with the deglycosylated recombinant protein from hCAT-1-expressing oocytes (Figure 8). In lysates not treated with N-glycosidase F a broader, more slowly migrating band at about 80–100 kDa was identified (Figure 8, last lane). Quantitative analyses of the hCAT-1-specific signal (normalized to β-tubulin) revealed no change in hCAT-1 protein levels by PMA treatment (100 nM) up to 4 h. After longer incubation times there was a moderate increase in hCAT-1 protein (up to 60% after 15 h). No change was seen in control cells treated with only 0.1% DMSO for the same periods of time (data not shown).

Discussion

In this study, we demonstrate that the activity of the human cationic amino acid transporter, hCAT-1, is downregulated by activation of PKC in either *Xenopus laevis* oocytes or EA.hy926 endothelial cells. *Xenopus laevis* oocytes offer the advantage of studying the activity of an individual carrier protein over-expressed in these cells, independent of the transcriptional and translational control of its expression. The inhibition of hCAT-1-mediated transport by the PKC-activating phorbol ester PMA occurred rapidly and was not observed with the inactive phorbol ester 4α-PDD, indicating an inhibitory action of PKC on the activity of the expressed hCAT-1 protein. A similar inhibitory effect of PMA can be observed when measuring hCAT-1-mediated L-arginine currents under voltage clamp conditions, showing that the PMA-induced transport inhibition cannot be attributed to changes in membrane potential (H. Nawrath, unpublished observation). Oocytes could not be incubated in 100 nM

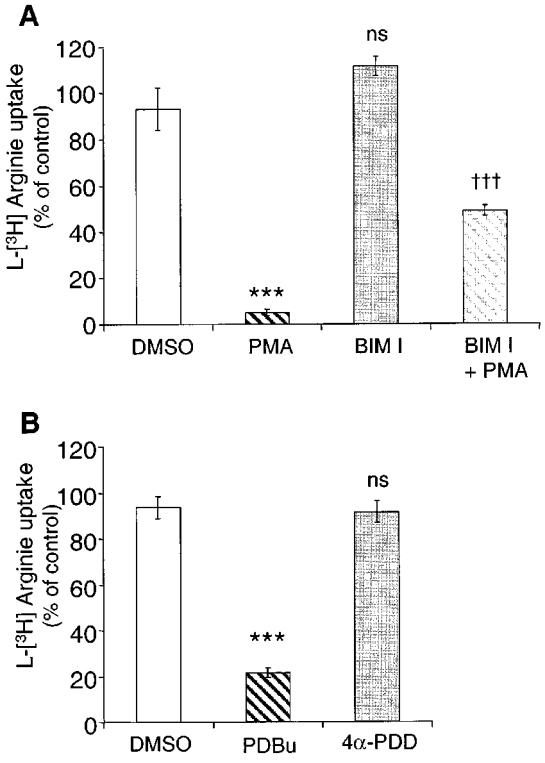

Figure 5 Downregulation of the L-arginine transport in EA.hy926 cells correlates with PKC activation (L-[^{3}H]-arginine uptake studies). Confluent cells were incubated for 4 h in Locke's solution supplemented with 100 μM unlabelled L-arginine and (A) 0.2% DMSO, 100 nM PMA, 1 μM BIM I, or 1 μM BIM I and 100 nM PMA; (B) 0.1% DMSO, 100 nM PDBu or 100 nM 4α-PDD. Locke's solution containing 100 μM L-[^{3}H]-arginine (5 μCi ml^{-1}) was then added and the cells were incubated for another 60 s at 37°C. The cells were then washed extensively, lysed and radioactivity and protein content of the lysates was determined. Data are expressed as per cent of the transport rate of untreated cells (2.63$\pm$0.16 pmol μg^{-1} protein min^{-1}), (means$\pm$s.e.mean, n=12). Asterisks indicate differences from DMSO-treated samples, ***: $P<0.001$, ns: not significant. Daggers indicate differences between cells treated with PMA only and with PMA plus BIM I, †††: $P<0.001$.

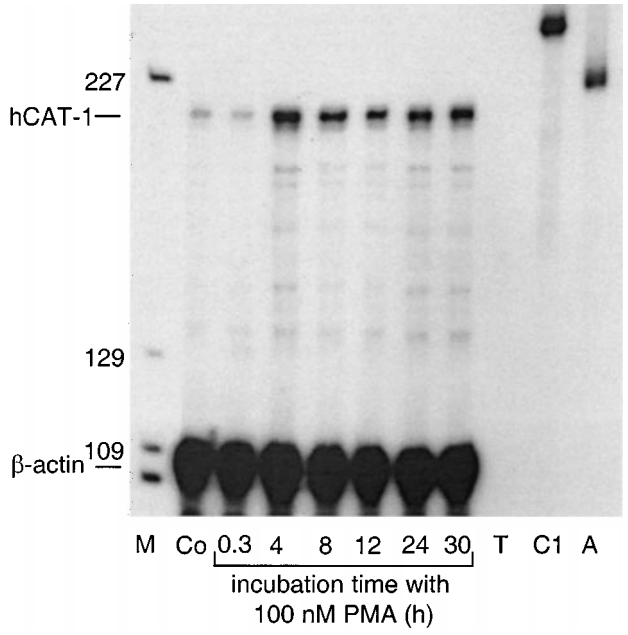

Figure 6 Upregulation of hCAT-1 mRNA in EA.hy926 cells by PMA (Ribonuclease protection analysis). Total mRNA was prepared from untreated EA.hy926 cells (Co) or from EA.hy926 cells exposed to 100 nM PMA (final DMSO concentration: 0.1 %) for 0.3, 4, 8, 12, 24 or 30 h. The RNAs were hybridized with antisense cRNA probes specific for hCAT-1, and for hβ-actin (as an internal control). After RNase treatment, the protected RNA fragments (hCAT-1: 201 nt, and hβ-actin: 109 nt) were separated on a 6% denaturing polyacrylamide gel. M: DNA size marker (pG12-Basic (Promega), restricted with *Hinf* I); T: t-RNA used as a negative control. C1, A: undigested probes for hCAT-1 (252 nt) and hβ-actin (222 nt) respectively. Similar results were obtained in four independent experiments.

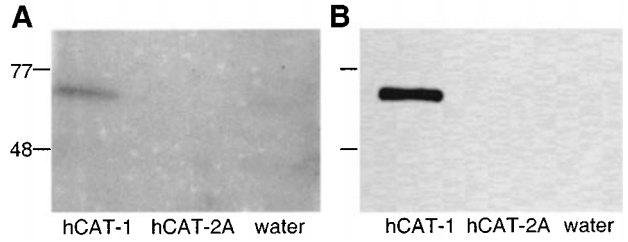

Figure 7 Characterization of the αhCAT-1/Kh5 immune plasma (Western blot analysis with lysates from *Xenopus laevis* oocytes). An immune plasma raised against the C-terminus of hCAT-1 (αhCAT-1/Kh5) was generated as described in experimental procedures. Lysates from *Xenopus laevis* oocytes (30 μg protein per lane) were separated by 10% SDS–PAGE (lane 1: oocytes expressing hCAT-1; lane 2: oocytes expressing hCAT-2A; lane 3: water-injected oocytes), blotted and the membranes incubated with the αhCAT-1/Kh5 immune plasma (A) or the affinity-purified αhCAT-1/Kh5 (B).

PMA for longer than 40 min, because thereafter, the cells underwent a marked change in morphology.

Consistent with the results in *Xenopus laevis* oocytes, activation of PKC by PMA or PDBu led to a decrease in L-arginine transport in EA.hy926 cells, whereas the inactive phorbol ester, 4α-PDD, had no effect. In addition, the PKC inhibitor BIM I markedly reduced the inhibitory effect of PMA. The inhibition of L-arginine transport by PMA was time- and concentration-dependent. The time course of inhibition correlates well with our previous results on PKC activation in these cells. Maximal transport inhibition was observed 1–4 h after addition of 100 nM PMA, when the PKC isoforms α and ε are trans-located to the plasma membrane (Li *et al*., 1998). Transport was restored to 50–70% of controls 20–30 h after PMA treatment, when both PKC isoforms are degraded (Li *et al*., 1998). In these PKC depleted cells, fresh PMA could not inhibit L-arginine transport one more time, indicating that the effect of PMA was mediated by activation of PKC. The absolute transport rate for 100 μM L-arginine varied in different experiments most likely due to differences in cell confluence previously shown to influence CAT-1 expression in different cell types (Yoshimoto *et al*., 1992). In general, freshly plated cells had a higher transport activity and were more sensitive to PMA inhibition than cells that were already confluent for 1 or 2 days. This can also explain, why the recovery of the L-arginine transport after long time treatment with PMA was only 50% of the initial transport rate of untreated cells, but 70% when compared to control cells cultured for the same time period (Figure 3B,C).

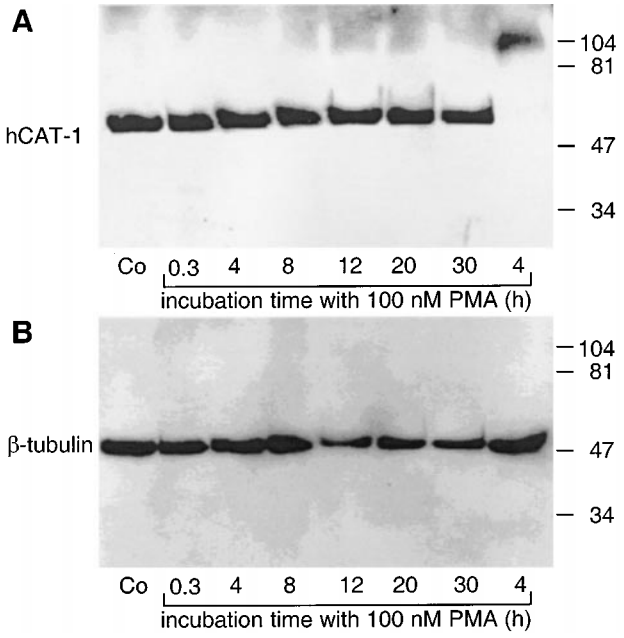

Figure 8 hCAT-1 protein expression in EA.hy926 cells after PMA treatment (Western blot analysis). To the medium of confluent EA.hy926 cells, 100 nM PMA was added and the cells incubated for 0.3, 4, 8, 12, 20, or 30 h with 100 nM PMA; Co: untreated control. Lysates in the first 7 lanes were treated with N-glycosidase F, the lysate in the last lane is the same as in lane 3 but not treated with N-glycosidase F. Forty μg protein per lane were separated by 10% SDS–PAGE, blotted and the membranes incubated with affinity-purified αhCAT-1/Kh5 antibody (A). The blot was then stripped and incubated with a monoclonal antibody to β-tubulin for standardization (B). The gel is representative of three experiments with similar results.

EA.hy926 cells exhibit a single transport component for L-arginine consistent with the transport properties of system y^+/hCAT-1 (Closs *et al*., 2000). In addition, expression of hCAT-1 mRNA, but not of any other CAT-isoform has been demonstrated in these cells. Therefore, hCAT-1 seems to be the only transport entity for L-arginine expressed in EA.hy926 cells. In this study, we demonstrate the expression of the hCAT-1 protein by Western blot analysis, using an affinity-purified antibody specific for hCAT-1. The hCAT-1 protein from EA.hy926 cells was glycosylated, indicating that hCAT-1 is correctly processed to be targeted to the plasma membrane (data not shown). The PMA-induced inhibition of the L-arginine transport was not accompanied by a decrease in hCAT-1 protein or in its glycosylation. Surprisingly, the expression of hCAT-1 mRNA was even increased several fold after a 4 h PMA treatment, when inhibition of transport was maximal. These results clearly demonstrate that assessment of the CAT mRNA expression is not sufficient to evaluate the amount of CAT protein in a given cell. As most studies in the past have exclusively relied on RNA analysis, the production of isoforms-specific antibodies, as demonstrated in this study, proves invaluable for the quantification of expression of the CAT-proteins. The increase in hCAT-1 mRNA may be a compensatory mechanism to restore the transport activity for cationic amino acids. Recently, it has been shown that amino acid starvation leads to a large increase in the 7.9 kb rat CAT-1 transcript in rat C6 glioma and NRK kidney cells, due to increased mRNA stability (Aulak *et al*., 1999). The hCAT-1 transcript is of similar size as the 7.9 kb rCAT-1 transcript, suggesting that it might also contain sequences responsible for stabilization of the mRNA during amino acid starvation. Therefore, PKC may have a direct effect on hCAT-1 expression by inducing transcription and/or stability of the hCAT-1 mRNA. The delayed and much more moderate increase in hCAT-1 protein, indicates that there may be translational control of the hCAT-1 mRNA. Alternatively, PKC may also reduce the half-life of the hCAT-1 protein resulting in no net increase of the protein in spite of a higher translational rate. As PKC-induced inhibition of hCAT-1-mediated transport is not accompanied by a reduction of hCAT-1 protein, hCAT-1 might either be internalized or inactivated by the action of PKC, as has been shown for other carrier proteins such as the human dopamine transporter (hDAT) (Zhu *et al*., 1997), and the rat glutamate/aspartate transporter (GLAST-1) (Conradt & Stoffel, 1997), respectively. Aside from consensus sequences for PKC, the amino acid sequence of hCAT-1 also contains consensus sequences for casein kinase II. Therefore, it remains to be elucidated if hCAT-1 is directly phosphorylated by PKC or if other kinases are involved. PKC may also act on a putative regulatory protein governing hCAT-1 activity. It is noteworthy that activation of PKC has opposite effects on L-arginine transport in different cell types. This may be due to different carrier proteins for cationic amino acids and/or different PKC isoforms expressed in these cells. In EA.hy926 cells, PMA treatment leads to the activation of PKCα and ε (Li *et al*., 1998). Further studies are required to investigate which of these isoforms is involved in the inhibition of the hCAT-1-mediated transport. Consistent with our results in EA.hy926 cells, a rapid decrease of system y^+ activity has been described in SK-Hep1 human hepatoma cells after PMA treatment (Bode *et al*., 1998). In contrast, in HUVEC (Pan *et al*., 1995b), Caco intestinal epithelial cells (Pan *et al*., 1995a) and peritoneal macrophages (Hortelano *et al*., 1993; Racke *et al*., 1998) PKC activation causes an increase in system y^+ activity. The carrier protein(s) involved in either down- or upregulation of system y^+ in the respective cells have not been identified. As several CAT proteins exhibit system y^+ activity (CAT-1, -2B and -3, for review see Closs & Gräf, 1999a), the differential effect of PKC activation in different cell types may reflect the expression of distinct CAT isoforms. Alternatively, different isoforms of PKC might be expressed in these different cell types.

In conclusion, our results demonstrate an inhibitory effect of PKC activation on the transport activity of hCAT-1 expressed in *Xenopus laevis* oocytes and endothelial cells. This inhibitory effect is independent of hCAT-1 mRNA or protein expression.

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