Migration of renal carcinoma cells is dependent on protein kinase Cδ via ß1 integrin and focal adhesion kinase

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Abstract. Migration and adhesion of tumor cells are essential prerequisites for the formation of metastases in malignant diseases. Protein kinase C (PKC) has been shown to regulate cell migration, adhesion and proliferation. In order to identify a connection between PKC isoforms and tumor progression in renal cell carcinoma (RCC), the influence of PKC isoforms on cell migration, adhesion and proliferation and possible influences of the activity of integrins and focal adhesion kinase (FAK) were analyzed in RCC cells. The experiments were performed in the RCC cell line CCF-RC1 after pre-incubation of the cells with the PKC inhibitors GF109203X, GÖ6976, RO31-8220 and rottlerin. Cell migration and adhesion were assessed through chemotaxis analysis and adhesion to an endothelial monolayer, respectively. Cell proliferation was analysed by a BrdU incorporation assay. The expression and activity of ß1 integrins and FAK were analysed by Western blot analysis. GF109203X reduced cell migration to 69%, the activity of ß1 integrins to 63% and FAK expression to 82% compared to untreated cells. Rottlerin reduced cell migration in a concentration-dependent manner to 36%, cell proliferation to 81%, expression and activity of ß1 integrins to 72 and 79%, and expression and activity of FAK to 56 and 76% of untreated cells, respectively. RO31-8220 also reduced the expression and activity of ß1 integrins as well as the expression of FAK to 84, 66 and 66% of untreated cells, respectively. GÖ6976 reduced the expression of FAK to 60% of untreated cells. Cell migration was only slightly reduced by GÖ6976 to 84% of untreated cells, and cell adhesion remained uninfluenced. These findings show a critical role of PKCδ in the regulation of tumor cell migration, which seems to be caused by affecting the expression and activity of ß1 integrins and FAK. These results can provide a basis for new strategies in preventing metastases of renal cell carcinoma.

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Abbreviations: BrdU, bromodeoxyuridine; ECM, extracellular matrix; FAK, focal adhesion kinase; HUVEC, human umbilical vein endothelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PKC, protein kinase C; RCC, renal cell carcinoma

Key words: renal cell carcinoma, protein kinase C isoforms, metastasis, integrin, focal adhesion kinase

Introduction

Renal cell carcinoma is a malignant tumor with a poor prognosis and high metastatic potential. Metastasis involves the separation from the primary tumor, migration into the extracellular matrix, blood vessel invasion, adhesion to endothelium and extravasation and growth in a secondary organ (1). During these steps, migration of tumor cells into the extracellular matrix (ECM) and adhesion to the endothelium are essential events in the formation of hematogenous metastases. Migration is a chemotactical process, caused by compounds of the ECM. Critically involved in this process as well as in cell adhesion are the integrin group of adhesion molecules, in particular the ß1 integrins (2,3). The binding of integrins to their ligands, such as compounds of the ECM like collagen or fibronectin, induces association with the actin cytoskeleton (4,5). Dependent on the state of cytoskeletal organization, this can lead to clustering of integrins into focal complexes including focal adhesion kinase (FAK). In this process ß1 integrins induce autophosphorylation and consequently activation of FAK on Tyr^{397}, which is responsible for promoting signal transduction downstream of FAK and influences cell motility (6).

The cell surface expression of ß1 integrins was shown to be positively correlated with increased metastatic potential and invasivity of renal tumor cells (7). Cells can rapidly change the function of integrins by altering the binding affinity of integrins for ligands as a result of inside-out signaling. The mechanism of inside-out signaling is a conformational change from the cytoplasmic domains in direction to the extracellular binding site in response to intracellular signaling events. Signaling molecules involved in inside-out signaling of integrins include - beneath G proteins, Ca^{2+}, phospholipase,

tyrosine kinase, CaM kinase II - the enzyme family of protein kinase C (PKC) (8-10). The activation pathway on integrins by PKC includes RACK (receptor for activated C kinase) which binds to the ß subunit of integrins (11). PKC modulation results in an alteration of the integrin avidity and affinity (12).

PKC-dependent activation of integrins includes phosphorylation of the cytoplasmatic domain of the integrin ß subunit (13). Phosphorylation of ß1 integrin cytoplasmic domains on Thr^{788} promotes inside-out receptor activation and focal contact accumulation. Phosphorylations on Thr^{788} and Thr^{789} are of critical importance for the function of integrins due to effects on the extracellular conformation of the receptor (14). In addition to the activity of integrins, PKC regulates the integrin expression on the cell surface (15,16) which leads to an altered cell migration capability (12). These results show a mutual activation pathway of PKC and the integrins.

PKC comprises a family of phospholipid-dependent serine/threonine protein kinases derived from different PKC genes and from alternative splicing of a single transcript (17). Up to 11 distinct family members have been discovered in mammalian cells, which are classified into Ca^{2+}-dependent conventional cPKC isoforms α, ßI, ßII and γ; Ca^{2+}-independent novel nPKCs δ, ε, η and θ; the atypical aPKCs λ/τ and ζ; and PKCμ/PKD, a Ca^{2+}-independent PKC with a unique substrate specificity which differs from the rest of the PKC isoforms (18). The expression patterns of PKC isoforms differ between different tissues and the subcellular distribution of the isoforms varies depending on cell type and physiological condition (19,20), so that an overexpression of the same PKC isoform may result in opposite biological effects, dependent on the cell type (21,22). In melanoma cells (23,24) and prostate tumor cells (25) PKCα seems to play a critical role in invasion, whereas in a metastatic clone of colorectal cancer cells PKCη is abundantly expressed (26). In contrast, an anti-metastatic effect of PKCζ was observed in rat prostate cancer cells (27). Also PKCε was described to be both positively (15,16,28) and negatively (29) involved in cell spreading on ECM and integrin-dependent migration. Until now, little is known about the isoform-specific role of PKC in integrin activation and metastasis of renal cancer. To answer this question, we investigated the influence of different isoform-specific PKC inhibitors on the metastatic behavior of renal carcinoma cells *in vitro*. Therefore, the involvement of PKC in the migration and adhesion of renal tumor cells *in vitro* as well as cell proliferation and the expression and activity of integrins and the focal adhesion kinase (FAK) was determined.

Materials and methods

Cells and cell culture. The human renal carcinoma cell line CCF-RC1 was used for all experiments. CCF-RC1 was isolated from a high grade pleomorphic renal cell carcinoma (nuclear grade 3) (30,31). The CCF-RC1 cell culture was maintained in RPMI-1640 with L-glutamine (Gibco, Eggenstein, Germany), supplemented with 10% fetal calf serum and 0.5% penicillin/streptomycin (Gibco). The cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 in air. Cells in the present study were used between passage 20 and 30.

For cell adhesion experiments, human umbilical vein endothelial cells (HUVECs) were isolated. Intact segments of 1- to 3-day-old umbilical cords were drained, and the remaining blood was rinsed from the umbilical vein with phosphate-buffered saline (PBS) via a blunt cannula attached at one end of the vessel. The open end of the cord was sealed, and the cord distended with 1 mg/ml collagenase II and incubated at room temperature for 30 min. The cord was massaged and cut, and the collagenase digest collected. The cells were washed, seeded into fibronectin-coated flasks (1 μg human fibronectin per cm^2 flask) and grown in Medium 199 supplemented with 20% fetal calf serum (FCS), 25 μg/ml heparin and 25 μg/ml endothelial cell growth supplement (ECGS). Cells were subcultured by trypsin-EDTA treatment and used until the fifth passage in the experiments.

Treatment of renal tumor cells with PKC inhibitors and assessment of viability. The renal tumor cells CCF-RC1 were incubated with the PKC inhibitors GF109203X, GÖ6976, RO31-8220 (concentrations of 1, 2 and 5 μM) and rottlerin (concentrations of 1, 5 and 10 μM) (all from Calbiochem, La Jolla, CA, USA) in serum-free medium for 30 min at 37˚C in a humidified atmosphere containing 5% CO_2 in air. The IC_{50} values of the inhibitors against PKC isoforms are listed in Table I. Serum-free culture medium without inhibitors served as a control. To exclude toxic effects of PKC inhibitors, renal tumor cells were incubated with PKC inhibitors as described above, each of them 4-fold. Afterwards, cells were washed with PBS and incubated with 0.4% trypan blue solution (Sigma, Deisenhofen, Germany). Fifty cells were evaluated, and the percentage of dead cells (blue colored) was determined. Furthermore, cell viability was assessed by the MTT Assay Kit (Sigma). In this assay the activity of living cells via mitochondrial dehydrogenases was measured. The yellow MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] was reduced to a blue formazan product by mitochondrial activity. Briefly, after incubation of the cells with PKC inhibitors, culture medium was replaced by MTT solution (0.5 mg/ml in phenol red-free medium) and incubated for 2 h at 37˚C. Afterwards, formazan crystals were dissolved by MTT solubilization solution and measured spectrophotometrically at a wavelength of 570 nm (reference wavelength 690 nm). All experiments were determined under conditions with a viability of not less than 95% of untreated cells.

Cell migration assay. A microchemotaxis chamber (Costar, Bodenheim, Germany) containing an upper and a lower space separated by a porous polycarbonate membrane (pore diameter 8 μm) was employed for the invasion experiments. The chamber was divided into 48 wells, resulting in an invasion unit with a surface of ~7.8 mm^2. Prior to the experiments, the porous membrane was coated with 0.1 mg/ml gelatine in 0.02 M acetic acid.

CCF-RC1 cells treated with PKC inhibitors and control cells were harvested with a 0.02% EDTA solution pH 7.2 (Gibco), washed with PBS and re-suspended in serum-free culture medium. In the experiments, the wells of the chemotaxis chamber were coated with 35 μl fibronectin (6 μg/ml) and covered with the polycarbonate membrane. The tumor cell suspension ($3x10^5$ cells/ml) was placed into the upper part

Table I. IC_{50} values for GF109203X, GÖ6976, RO31-8220 and rottlerin for the inhibition of PKC isoforms.[a]

Inhibitors	PKC isoforms								
	α	ßI	ßII	γ	δ	ε	η	ζ	μ
GF109203X (nM)	14.0	18.0	16	20	210	132		5,800	2,000
GÖ6976 (nM)	2.3	6.2			>10,000	>10,000	685	>10,000	20
RO31-8220 (nM)	5.0	24.0	14	27		24			
Rottlerin (μM)	30.0	42.0		40	3-6	100	82	100	

[a]Goekjian and Jirousek (36) and Gschwendt *et al* (50).

of the chamber. After an incubation period of 16 h at 37˚C in a humidified atmosphere containing 5% CO_2 in air, cells that did not pass the polycarbonate membrane were removed from the upper side of the porous membrane by washing with PBS and by mechanical removal with a rubber policeman. The membrane was dried for 30 min and fixed in methanol. Afterwards, the nuclei and cytoplasm were specifically stained with hemacolor (Merck, Darmstadt, Germany) and embedded on a glass slide coated with immersion oil. The number of invasive tumor cells was evaluated by a microscopic test raster ocular (Zeiss, Oberkochen, Germany, x400 magnification) (32). For a single determination, ten different views per well with a combined membrane surface of 2.5 mm^2 were evaluated. The experiments were performed 4- to 8-fold and repeated four times.

Cell adhesion assay. To analyze the adhesion of renal tumor cells to an endothelial monolayer, HUVECs ($1.5x10^4$ cells/well) were seeded into a 96-well plate coated with 1.5 mg/cm^2 gelatine. Cells were allowed to grow to a confluent monolayer within three days. PKC inhibitor-treated and control CCF-RC1 cells were labeled by 10 μM BrdU (Bromodeoxyuridine) for 30 min at 37˚C. A cell suspension containing 10^4 cells was added to the endothelial cell layer under the presence of the particular PKC inhibitor and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 in air. After 2 h, non-adherent cells were removed by washing with PBS. To normalize the level of BrdU per cell, a defined number of 10^4 BrdU-labeled tumor cells were centrifuged in parallel into a 96-well plate. The relative amount of BrdU per well was quantified as described in the next section. Each experiment was performed 8-fold and repeated three times.

Analysis of cell proliferation. To study the effect of PKC inhibitors on proliferation, a colorimetric BrdU incorporation assay (Roche, Mannheim, Germany) was performed. Each $3x10^3$ cells were seeded into a 96-well plate and treated in triplicate by PKC inhibitors in serum-free medium. BrdU solution (10 μM) was added to the cells and incubated for 2 h at 37˚C in a humidified atmosphere containing 5% CO_2 in air. After removing the culture medium, the cells were fixed, and the DNA was denatured in one step by adding fixDenat solution. Incorporated BrdU was detected by an anti-BrdU-POD antibody. The immune complex was detected by a subsequent substrate reaction and quantified by measuring the absorbance at 450 nm (reference wavelength 690 nm).

Western blot analysis of overall and phosphorylated ß1 integrins and FAK. Protein extracts were prepared from the PKC inhibitor-treated renal carcinoma cell line CCF-RC1 by hot (95˚C) lysis buffer (120 mM Tris/HCl pH 6.8, 11.5% glycerol, 3.3% SDS, 3.3% mercaptoethanol). Protein extract was separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of 7.5% polyacrylamide and transferred by semi-dry blotting onto polyvinylidene fluoride membranes (PVDF, Immobilon P, Millipore, Bedford, MA, USA) for Western blotting (33). The membrane was blocked in Roti-block blocking solution (Roth, Karlsruhe, Germany) for detection of ß1 integrins and FAK and in TBS, 0.1% Tween-20, 5% nonfat dry milk powder for detection of phosphorylated antigens for 1 h. The primary antibodies were incubated overnight at 4˚C in Roti-block blocking solution. The mouse monoclonal antibodies mouse anti-ß1 integrins, rabbit anti-phosphorylated ß1 integrins ($Thr^{788/789}$) (both from Biosource, Camarillo, CA, USA), rabbit anti-FAK (Upstate, Lake Placid, NY, USA) and mouse anti-phosphorylated FAK (Tyr^{397}) (Chemicon Hofheim, Germany) were diluted 1:1000. A mouse anti-ß-actin-specific antibody (Sigma) served as control. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and diluted 1:2000 for 1 h at room temperature. The bound antibodies were visualized by an enhanced chemiluminescence (ECL) detection system using Fuji medical X-ray film, and the Western blot membrane was stained with Coomassie brilliant blue (Sigma) (34). The amount of antigen was calculated by computer-aided integration of the band after subtraction of the background using ImageJ software (35).

Statistical analysis. For statistical confirmation, three or four independent adhesion and migration experiments, each 4- to 8-fold, were performed respectively. A mean value and a standard error were calculated from the results.

Results

Cell migration after treatment with PKC inhibitors. In a first step to analyze the role of PKC isoforms in processes of metastases, we investigated the influence of the PKC inhibitors GÖ6976, GF109203X and RO31-8220 on migration of CCF-RC1 cells. Cells were treated with the inhibitors in a concentration of 2 μM for 30 min, and the migration was quantified in the Boyden chemotaxis chamber. Treatment with GF109203X, which inhibits PKCα, ßI, ßII, γ, δ and ε,

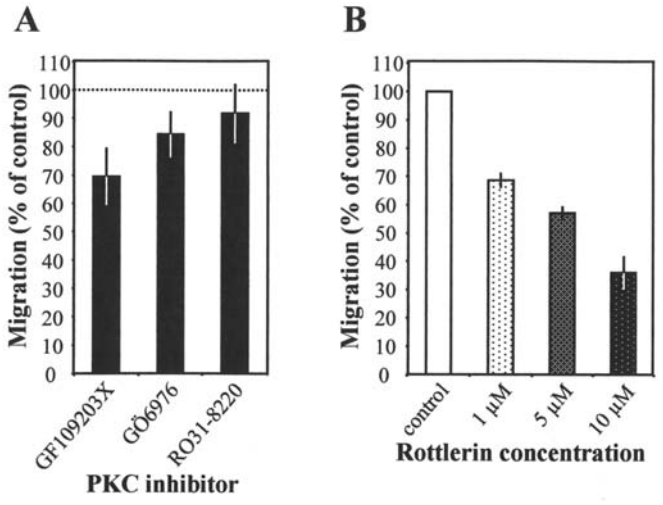

Figure 1. Migration of CCF-RC1 cells after treatment (A) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (B) with rottlerin in concentrations between 1 and 10 μM. As chemotactic agents, fibronectin (6 μg/ml) was applied. The migration value was determined as a percentage of the migration of untreated cells. The columns and bars represent the mean value and standard error.

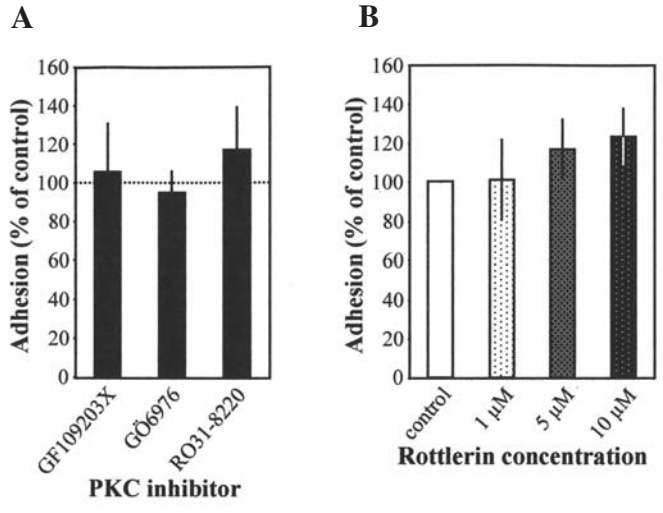

Figure 2. Cell adhesion of CCF-RC1 cells to a monolayer of human umbilical endothelial cells (HUVECs) after treatment (A) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (B) with rottlerin in concentrations between 1 and 10 μM. The adhesion value was determined as a percentage of the adhesion of untreated cells. The columns and bars represent the mean value and standard error.

resulted in a 31% inhibition of migration to 69% of untreated cells. In contrast, treatment with GÖ6976, which inhibits PKCα, ßI and μ, or RO31-8220, which inhibits PKCα, ßI, ßII, γ and ε (36), affected the migration of CCF-RC1 cells less intensely, namely ~16 or 8%, respectively (Fig. 1A).

The only PKC isoform, which is inhibited by GF109203X (strong inhibitory influence on migration) but not by GÖ6976 or RO31-8220 (both less intense influence on migration) is PKCδ. In order to obtain more direct evidence for PKCδ being involved in migration control, we treated CCF-RC1 cells with the PKCδ inhibitor rottlerin. Treatment with rottlerin for 30 min resulted in a reduction of migration in a concentration-dependent manner. Incubation of renal tumor cells with 1, 5 or 10 μM rottlerin led to a reduction of migration to 69, 57 and 36% of untreated cells, respectively (Fig. 1B).

Cell adhesion of PKC inhibitor-treated renal tumor cells to endothelial cells. In the next approach, we investigated the influence of the PKC inhibitors used for migration analysis for the adhesion of renal tumor cells to endothelial cells. The CCF-RC1 cells were treated with PKC inhibitors GF109203X, GÖ6976, RO31-8220 and rottlerin as described, and the adhesion of these cells to a monolayer of HUVECs was determined. We observed no inhibitory effect in cell adhesion after treatment with GF109203X, GÖ6976 and RO31-8220 (Fig. 2A). The enhanced adhesion after treatment with RO31-8220 of 17% should be interpreted with caution, since the standard error is very large. Rottlerin induced an enhancement of adhesion in a concentration-dependent manner to 23% of untreated cells (Fig. 2B).

Cell proliferation after treatment with PKC inhibitors. To assess the influence of PKC inhibition on cell proliferation, CCF-RC1 cells were incubated with the PKC inhibitors GF109203X, GÖ6976 and RO31-8220 in concentrations as described. Proliferation was determined by BrdU incorporation. GF109203X, GÖ6976 and RO31-8220 did not affect the cell proliferation or only marginally of ~3, 6 and 8%, respectively. Proliferation of CCF-RC1 upon rottlerin treatment was reduced between 7 and 20% (Fig. 3).

Influence of PKC inhibitors on the expression and phosphorylation of ß1 integrins. Since our former investigations showed a strong involvement of ß1 integrins in the migration of CCF-RC1 cells (2), the expression of overall and phosphorylated ß1 integrins was determined by Western blot analysis and quantified by computer-aided densitometry. After treatment of CCF-RC1 cells with the PKC inhibitor

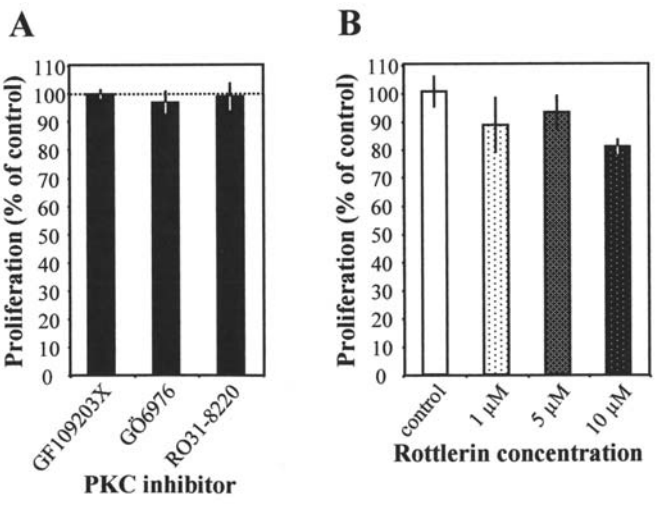

Figure 3. Cell proliferation of CCF-RC1 cells after treatment (A) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (B) with rottlerin in concentrations between 1 and 10 μM. The proliferation value was determined as a percentage of the proliferation of untreated cells. The columns and bars represent the mean value and standard error.

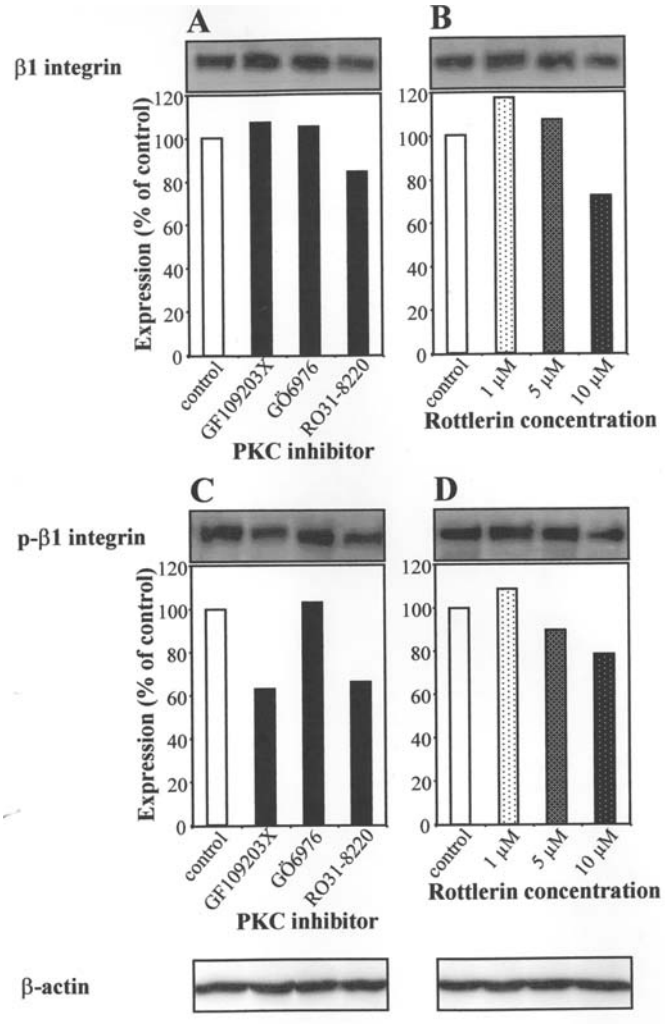

Figure 4. Expression of ß1 integrins in CCF-RC1 cells after treatment (A) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (B) with rottlerin in concentrations between 1 and 10 μM, and expression of phosphorylated ß1 integrins (Thr$^{788/789}$) (p-ß1 integrin) in CCF-RC1 cells after treatment (C) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (D) with rottlerin in concentrations between 1 and 10 μM, determined by Western blot analysis. As a loading control, ß-actin was analysed in parallel. The columns represent the amount of antigen determined by computer-aided integration of the band after subtraction of the background.

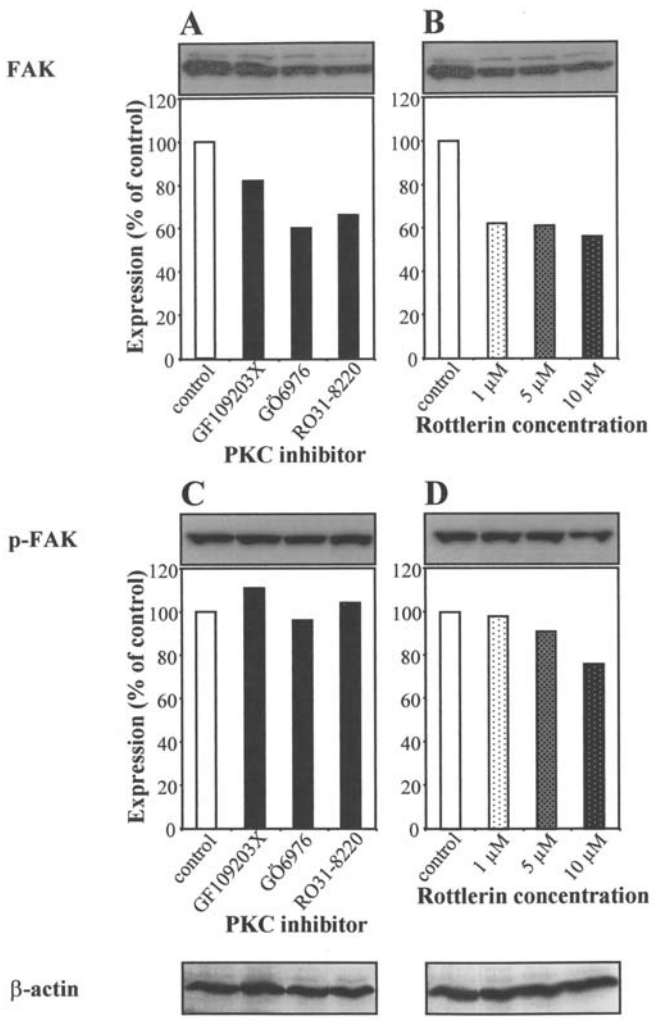

Figure 5. Expression of FAK in CCF-RC1 cells after treatment (A) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (B) with rottlerin in concentrations between 1 and 10 μM, and expression of phosphorylated FAK (Tyr^{397}) (p-FAK) in CCF-RC1 cells after treatment (C) with the PKC isoform-specific inhibitors GF109203X, GÖ6976 and RO31-8220 in a concentration of 2 μM, and (D) with rottlerin in concentrations between 1 and 10 μM, determined by Western blot analysis. As a loading control, ß-actin was analysed in parallel. The columns represent the amount of antigen determined by computer-aided integration of the band after subtraction of the background.

GF109203X, the expression of phosphorylated and therefore active ß1 integrins was reduced to 63% of untreated cells, whereas the overall expression of ß1 integrins was not affected. RO31-8220 inhibited the expression of overall and phosphorylated ß1 integrins to 84 and 66% of untreated cells, respectively. GÖ6976 did not influence the expression of overall or phosphorylated ß1 integrins. Rottlerin reduced the expression and phosphorylation of ß1 integrins only in a concentration of 10 μM to 72 and 79% of untreated cells, respectively (Fig. 4).

Influence of PKC inhibitors on the expression and phosphorylation of FAK. Expression and phosphorylation of FAK, a molecule involved in the formation of adhesion plaques on the intracellular section of integrins, were determined as described for ß1 integrin expression. After cell treatment with the PKC inhibitor GF109203X, GÖ6976 and RO31-8220, the expression of FAK was reduced to 82, 60 and 66% of untreated cells, respectively. These inhibitors did not influence the expression of phosphorylated FAK. Rottlerin also reduced the expression of FAK in a concentration-dependent manner to 56% of untreated cells. Additionally rottlerin reduced the expression of phosphorylated and therefore active FAK in a concentration-dependent manner to 76% of untreated cells (Fig. 5).

Discussion

Migration and adhesion of tumor cells are the most important prerequisites for formation of metastasis. We investigated the influence of different PKC isoforms on the regulation of migration and adhesion of the renal carcinoma cell line CCF-

RC1. The cells were incubated with PKC isoform-specific inhibitors GF109203X (inhibits PKCα, ßI, ßII, γ, δ and ε), GÖ6976 (inhibits PKCα, ßI and μ) and RO31-8220 (inhibits PKCα, ßI, ßII, γ and ε) (36) in concentrations which had no toxic effect on the cells, as determined by an MTT test and trypan blue staining. Afterwards, we quantified cell migration in a Boyden microchemotaxis chamber and cell adhesion to endothelial cells. Of the three PKC inhibitors GF109203X, GÖ6976 and RO31-8220, only GF109203X treatment resulted in a clearly reduced migration (69% of untreated cells). This reduced migration might have been caused by the additionally observed reduced expression of phosphorylated and therefore active ß1 integrins to 63% of untreated cells. In former studies, we detected ß1 integrins as the main mediator of CCF-RC1 migration (2). The only PKC isoform which was inhibited by GF109203X, but not by GÖ6976 or RO31-8220, was PKCδ. This leads to the assumption that PKCδ is involved in the regulation of migration of the renal cancer cells CCF-RC1. These results were confirmed by pre-treatment of the cells with the more specific PKCδ inhibitor rottlerin. Since PKCδ is less sensitive to rottlerin (IC_{50} 3-6 μM) than to GF109203X (210 nM, Table I), rottlerin was applied at a higher concentration (10 μM instead of 2 μM). Treatment with rottlerin caused a reduction of migration to a level of 36% of migration of untreated cells with an inhibition of expression and activity of ß1 integrins to 72 and 79%, respectively. Furthermore, the downstream kinase of integrins, FAK, was also reduced in expression and activity after treatment of the cells with rottlerin to 56 and 76% of untreated cells, respectively. FAK is known to regulate not only cell motility but also cell proliferation via p27 (37), so that the reduced activity of FAK after cell treatment with rottlerin should additionally result in a reduced cell proliferation. Indeed, cell proliferation was reduced down to 81% of untreated cells. Thus, we can deduce that PKCδ regulates cell migration and, at a lower level, cell proliferation, possibly by regulation of the expression of ß1 integrins and FAK.

PKCδ is known to be involved in regulation of the cell cycle and apoptosis. It induces cell cycle arrest in a variety of cell types inhibiting the expression of cyclin D1 and cyclin E and an up-regulating of p27 (38-40). Furthermore, PKCδ has been shown to activate DNA-PK (DNA-dependent protein kinase) (41), to antagonize the Jak-STAT pathway (42) and to inhibit phospholipase D, an antagonist of Raf (36). On the other hand, an inhibition of PKCδ induced apoptosis in B chronic lymphocytic leukemia cells, whereas in healthy B cells a more anti-apoptotic effect was mentioned (43). Also in glioma cells an inhibition of PKCδ by rottlerin led to a reduced activity of ERK and Akt and inhibited cell proliferation (44). This shows a strong cell-specific effect of PKCδ.

In addition to its regulation of cell proliferation and apoptosis, PKCδ was shown to influence metastasis. PKCδ-overexpressing melanocytes showed an increased metastatic capacity (45), and studies by Kiley *et al* (46) and Oh *et al* (47) demonstrated that PKCδ is important for cytoskeleton-regulated processes in metastasis of mammary tumors and epithelial cells. An inhibition of PKCδ resulted in a reduced migration of glioma cells (44). These results confirm, along with our finding, that PKCδ regulates the cell migration of renal cancer cells in a chemotactic migration assay.

In a study concerning renal cell carcinoma, Engers *et al* suggested a particular activation of PKCα and PKCε in highly invasive renal cancer cell lines and hence a possible role of these PKC isoforms in invasiveness (48). Notably, PKCδ was not expressed in the investigated cell lines, so that a possible role of this PKC isoform was not established. In CCF-RC1 cells used in our study, all PKC isoforms were constitutively expressed with the exception of PKCγ and θ (15). This *in vitro* PKC expression pattern is analogous to PKC isoform expression on surgically removed RCC lesions (49), suggesting that expression of PKC isoforms in the RCC cell line CCF-RC1 corresponds well to the *in vivo* situation, which is not the case for several other renal tumor cell lines (48).

The PKC inhibitor RO31-8220 also reduces the expression of overall FAK and reduces expression of ß1 integrins and of phosphorylated ß1 integrins in nearly the same manner as GF109203X and rottlerin. Since treatment of CCF-RC1 cells with RO31-8220 over 16 h reduced the expression of ß1 integrins on the cell surface without influencing cell migration (15), it would seem that in addition to PKCδ, other PKC isoforms like PKCε influence the expression of these molecules without regulating cell migration. PKCε is inhibited by RO31-8220 as well as by GF109203X (Table I), so that the observed reduced phosphorylation of ß1 integrins can be attributed to an inhibition of PKCδ and/or PKCε, while in contrast to PKCε, PKCδ also influences cell migration. This shows that several PKC isoforms are involved in the regulation of tumor cell migration and the expression and activation of integrins.

In conclusion, the results presented here indicate that protein kinase Cδ may be involved in the regulation of chemotactical tumor cell migration. The influence in cell migration seems to be caused by affecting the expression and activation of ß1 integrins, receptors of extracellular matrix compounds, and also of focal adhesion kinase, a signal mediator downstream of the integrins.

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