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Vitronectin-dependent invasion of epithelial cells by *Neisseria gonorrhoeae* involves α_v integrin receptors

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Abstract Binding of vitronectin (VN) to *Neisseria gonorrhoeae* expressing the heparan sulfate proteoglycan (HSPG) specific Opa_{50} protein was recently shown to trigger bacterial internalization into distinct epithelial cell lines. We have investigated the role of VN-binding integrin receptors and protein kinase C (PKC) in VN-triggered bacterial uptake. Blocking integrin function by RGDS peptides or by antibodies specific to $\alpha_v\beta_5$ or $\alpha_v\beta_3$ resulted in an abrogation of VN-triggered bacterial internalization. Moreover, inhibitors of PKC were found to block VN-triggered uptake. The essential role of α_v integrins and the presumable involvement of PKC in VN-triggered gonococcal uptake are discussed.

Key words: *Neisseria gonorrhoeae*; Invasion; Vitronectin; Integrin; Protein kinase C

1. Introduction

The genus *Neisseria* encompasses Gram-negative, diplococcal bacteria that successfully colonise human mucosal tissues. *Neisseria gonorrhoeae*, the gonococcus, causes a sexually transmitted disease, gonorrhoea, which is clinically characterized by an intense inflammatory reaction resulting in the formation of a purulent discharge. Occasionally, *N. gonorrhoeae* infection may remain asymptomatic or may spread systemically (disseminated gonococcal infection) [1]. At the molecular level, gonococcal infection of human mucosal cells is thought to involve a sequential interaction between bacterial adhesins and their receptors on the host cell surface. The gonococcal pilus mediates the initial binding to human epithelial cells [2,3] while more intimate binding interactions are established by members of the colony opacity-associated Opa protein family [4,5]. While most of the eleven distinct gonococcal Opa variant proteins in strain MS11 bind to members of the carcinoembryogenic antigen receptor family (CD66) expressed on distinct cell types [6–9], only one member, Opa_{50}, is capable of mediating both intimate adherence to and invasion of cultured epithelial cells [5]. This binding and internalization of Opa_{50}-expressing gonococci is mediated by cell surface-associated heparan sulfate proteoglycans (HSPGs) [10–12]. HSPG-dependent bacterial internalization has been shown to occur by at least two alternative mechanisms. Invasion into some epithelial cell lines, e.g. Chang conjunctiva cells, occurs irrespective of the presence of serum but depends on the activation of phosphatidylcholine-dependent phospholipase C (PC-PLC) and acidic sphingomyelinase (ASM) which results in the generation of the second messengers diacylglycerol (DAG) and ceramide, respectively [13]. In many other epithelial cell lines, e.g. HeLa, this signaling pathway appears to be less prominent, while serum was shown to allow an alternative pathway of HSPG-dependent invasion. The invasion stimulating factor in serum has been identified as the extracellular matrix protein vitronectin (VN), which was shown to specifically bind to Opa_{50}-expressing gonococci [14,15]. The physiological role of VN involves the regulation of cell adherence and cell migration [16]. Likewise, as with some other extracellular matrix (EM)-proteins, VN contains an RGD peptide motif capable of binding to heterodimeric cell surface receptors of the integrin family. The integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$, and $\alpha_{IIb}\beta_3$ have been shown to specifically serve as VN-receptors [16,17]. Endocytosis of VN can be mediated either by $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. Internalization of VN by integrin $\alpha_v\beta_3$ further requires ligation of integrin $\alpha_5\beta_1$ [18]. In contrast, $\alpha_v\beta_5$ integrin receptor-mediated endocytosis of VN is dependent on signaling by protein kinase C (PKC) which appears to be activated by co-ligation of heparan sulfate proteoglycans [19,20]. Pathogens capable of interacting with these VN receptors, either directly or through binding of VN, may subvert the stimulated endocytosis processes in order to gain access to the cell interior. Direct binding of adenovirus to both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors was indeed shown to result in virus internalization [21]. In the present study, we tested for the role of these receptors, and began to dissect the intracellular signaling processes which may be associated with the gonococcal host cell interaction. We demonstrate that VN-triggered internalization of gonococci into epithelial cells is dependent on the VN-binding integrins $\alpha_v\beta_5$ and to a lesser extent also $\alpha_v\beta_3$, and further appears to require signaling by PKC. Despite the fact that many bacterial pathogens were previously demonstrated to bind VN, this is, to our best knowledge, the first demonstration of a VN-dependent signaling process being involved in bacterial internalization into host cells.

2. Materials and methods

2.1. Cell culture

HeLa (ATCC CCL2) derived from a cervical carcinoma, Hec1B (ATCC HTB 113) derived from endometrial adenocarcinoma, Chang (ATCC CCL20.2) derived from conjunctiva, and ME180 (ATCC HTB33) derived from cervix carcinoma were maintained in RPMI 1640 tissue culture medium (Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal calf serum (FCS). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Bacteria

Derivatives of the *N. gonorrhoeae* strain MS11 were used in this

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study. The mutant N300 contains an insertion of a chloramphenicol acetyltransferase gene in the *opaC* locus which encodes the invasion-mediating Opa_{30} protein and therefore served as an invasion deficient control strain [5]. The invasive strain N303 is N300 containing a plasmid which encodes the Opa_{50} protein, the phase-invariant counterpart of the chromosomally encoded Opa_{30} [5]. Both strains are non-piliated (P^-). Neisserial strains were grown at 37°C with 5% CO_2 in GC-agar base (Life Technologies, Paisley, UK) supplemented with 1% VitaleX (BBL) and antibiotics when appropriate.

2.3. Reagents and antibodies

Human VN, Quantum Simply Cellular Microbead Kit, peptides and inhibitors were purchased from Sigma (Deisenhofen, Germany). Monoclonal antibodies (mAbs) anti-$\alpha_v\beta_3$ (clone LM609), anti-$\alpha_v\beta_5$ (clone P1F6) and anti-$\alpha_5\beta_1$ (clone JBS5) were obtained from Chemicon International (Temecula, CA). The anti-HLA mAb (clone DDII) which served as irrelevant isotype matched control antibody and the fluorescein (DTAF) conjugated goat anti-mouse $F(ab)_2$ were obtained from Dianova (Hamburg, Germany).

2.4. Infection experiments

Cell association assays and gentamicin invasion assays were carried out as described before [15]. In some experiments, VN was added for a second time after 4 h of incubation to further trigger invasion. For inhibitor studies, staurosporine and calphostin C were preincubated with the cells for 15 min before adding the bacteria. In antibody blocking experiments, cells were preincubated with antibodies for 1 h before the addition of bacteria.

2.5. Flow cytometry

Cells were harvested with PBS containing 0.5 mM EDTA. After washing with PBS/0.5% BSA, 2×10^5 cells or 30 μl microbeads were incubated with 2 μg of various mAbs for 2 h on ice. Cells were washed two times with PBS/0.5% BSA and treated with the secondary antibody (1:50 dilution of DTAF conjugated goat anti-mouse IgG). After washing twice with PBS/0.5% BSA, cells were resuspended in 300 μl PBS/0.5% BSA supplemented with 5 μg/ml propidium iodide and analysed using a flow cytometer (FacsScan, Becton Dickinson, San Jose, CA). Background staining was assessed by incubation with an irrelevant, isotype-matched anti-HLA antibody. Antibody binding capacity (ABC) units, an arbitrary measurement of antigen density on the cell surface, were determined by the Quantum Simply Cellular Kit (Sigma, Deisenhofen, Germany).

3. Results

3.1. VN-triggered invasion of HeLa cells by Opa_{50}-expressing gonococci is inhibited by RGDS peptide

VN contains an RGD sequence capable of mediating interaction with several integrin receptors on the cell surface [22,23]. To test if integrins may be involved in VN-triggered invasion, invasion as well as cell-association assays were performed in the presence of the inhibitory RGDS peptide or the non-blocking control peptide RGES. As a source of VN, we have used in this and all subsequent experiments both 5% of whole fetal calf serum (FCS) or 2.5 μg/ml of purified human vitronectin (VN) in parallel assays. Compared to BSA as a negative control, invasion of Opa_{50}-expressing gonococci was consistently increased about 10-fold by FCS. Invasion was generally increased in a range of 3- to 6-fold depending on the batch of VN used. Bacterial adherence was similar under all conditions. In the presence of 2 mM RGDS peptide, invasion of Opa_{50}-expressing gonococci into HeLa cells was completely abrogated, while invasion was not significantly affected by 2 mM of the RGES control peptide (Fig. 1, black bars). Blockage of invasion by the RGDS peptide was concentration-dependent with maximum inhibition resulting at concentrations of 2 mM and higher (data not shown). Cell association assays were performed to exclude that the inhibitory effect of the RGDS peptide is due to an inhibition of bacterial adherence. Concentrations of 2 mM of either RGDS or RGES peptides did not significantly affect the adherence of Opa_{50}-expressing gonococci in the presence or absence of either FCS or VN (Fig. 1, gray bars). This is consistent with the fact that Opa_{50}/HSPG-interactions mediate bacterial adherence to the epithelial cell surface [10], however, invasion is negligible in the absence of VN-mediated interactions.

3.2. Identification of integrins involved in VN-triggered invasion of HeLa cells by Opa_{50}-expressing gonococci

The integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_{IIb}\beta_3$ are the major known receptors for VN [17], and $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins have been implicated in the endocytosis of VN [18–20]. In order to test if the latter two integrins contribute to VN-triggered invasion, we have performed invasion assays in the presence of monoclonal antibodies (mAbs) specific for these receptors and known to block binding to their natural ligands [21,24]. VN- or FCS-triggered invasion of HeLa cells by gonoxcocci was strongly inhibited in the presence of anti-$\alpha_v\beta_5$ mAbs (approximately 6-fold) and moderately inhibited in the presence of anti-$\alpha_v\beta_3$ mAbs (approximately 2-fold for VN vs. 3-fold for FCS). In contrast, anti-$\alpha_5\beta_1$ mAbs known to block

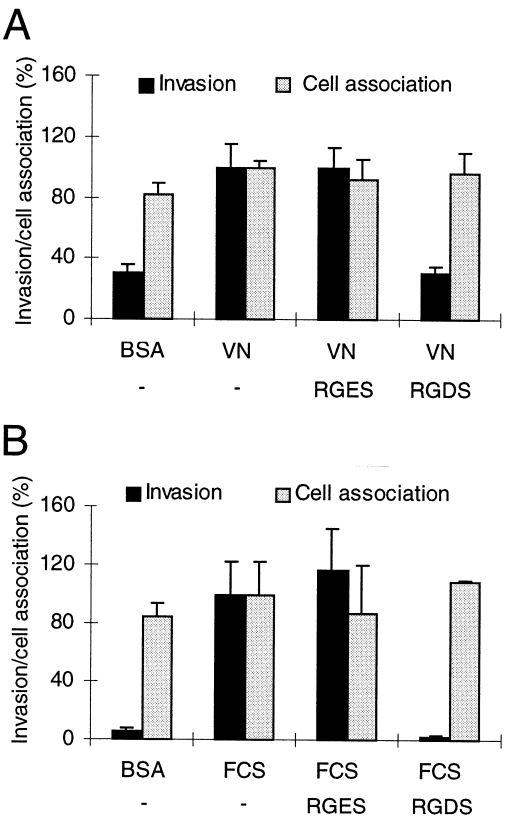

Fig. 1. Inhibition of vitronectin-triggered entry of Opa_{50}-expressing gonococci into HeLa cells in the presence of integrin function blocking peptides. Bacterial invasion assays or cell association assays were performed in the presence or absence of (A) 2.5 μg/ml human vitronectin (VN) or (B) 5% fetal calf serum (FCS) for 6 h. RGDS or RGES peptides were added to a final concentration of 2 mM as indicated. The invasion (black bars) or cell association (gray bars) of (A) VN or (B) FCS in the absence of further supplements was set to 100% and all other data were expressed in percentages of these reference values. The bars and error bars represent the mean and standard deviation of triplicate samples obtained in one representative experiment.

the binding of $\alpha_5\beta_1$ to its ligand fibronectin did not significantly affect gonococcal invasion (Fig. 2). All three tested integrins were found to be expressed on the surface of HeLa cells as determined by flow cytometry (Fig. 3).

3.3. VN-dependent invasiveness of Opa_{50}-expressing gonococci correlates with the level of surface expression of α_v integrins in different cell lines

The level of VN-triggered invasion varies significantly in different cell lines [15]. In order to test if VN-dependent invasiveness may correlate with VN-receptor expression, we have performed in parallel invasion assays and flow cytometric density measurements of the surface expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins on a panel of epithelial cell lines. Interestingly, we found increased expression of these integrins in cell lines exhibiting VN-dependent invasiveness (Hec1-B and HeLa) compared to cell lines which do not show increased invasion in the presence of VN (Chang and Me-180, Fig. 3). Integrin $\alpha_5\beta_1$, which served as a control, was found to be expressed at roughly equal levels by all cell lines tested.

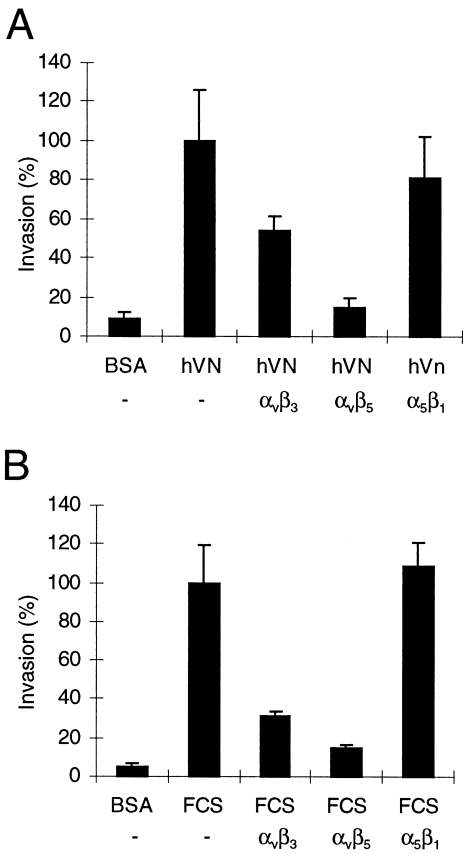

Fig. 2. Inhibition of vitronectin-triggered entry of Opa_{50}-expressing gonococci into HeLa cells in the presence of anti-integrin antibodies. Bacterial invasion assays were performed in the presence or absence of (A) 2.5 μg/ml human vitronectin (VN) or (B) 5% fetal calf serum (FCS) for 6 h. The mAbs directed against various integrins as indicated were added in a final concentration of 40 μg/ml. The invasion mediated by (A) VN or (B) FCS in the absence of further supplements was set to 100% and all other data were expressed in percentages of these reference values. The bars and error bars represent the mean and standard deviation of triplicate samples obtained in one representative experiment.

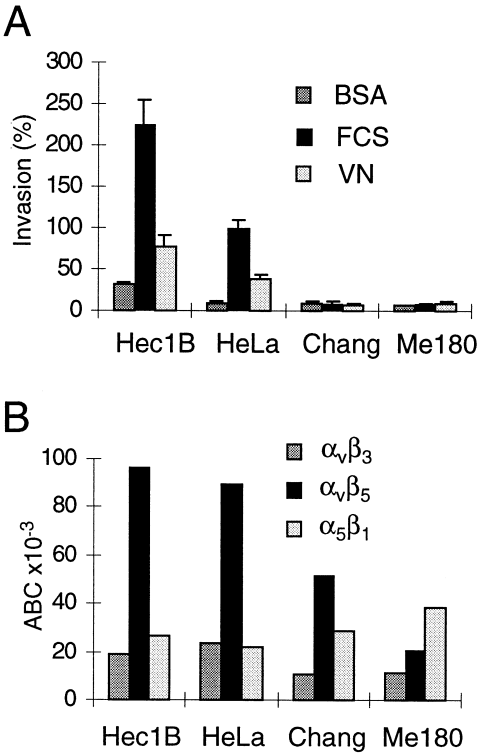

Fig. 3. Comparison of vitronectin-triggered invasiveness of Opa_{50}-expressing gonococci and integrin receptor expression for a panel of epithelial cell lines. A: Invasion assays were performed in the presence or absence of 2.5 μg/ml human VN (VN) or 5% fetal calf serum (FCS). The bars and error bars represent the mean and standard deviation of triplicate samples of one representative experiment. B: Integrin receptor density was measured with specific mAbs by flow cytometry as described in Section 2. The bars indicate antibody binding capacities (ABC units) for the indicated antibodies obtained in one representative experiment.

3.4. VN-mediated invasion of HeLa cells by Opa_{50}-expressing gonococci is blocked by PKC inhibitors

Previous studies have indicated that $\alpha_v\beta_5$-mediated endocytosis of VN is dependent on signaling by PKC [20]. To examine the role of PKC for VN-triggered invasion, invasion assays were performed in the presence of the serine/threonine kinase inhibitor staurosporine or the PKC-specific inhibitor calphostin C. VN- or FCS-dependent invasion was inhibited by staurosporine in a concentration dependent manner (Fig. 4A,B), except for a slight increase in FCS- but not VN-triggered invasion which was repeatedly seen at low inhibitor concentrations (100 nM or less). This effect may reflect the slight increase in bacterial adherence resulting from staurosporine treatment (Fig. 4A,B). VN- or FCS-triggered gonococcal invasion was also blocked by calphostin C in a concentration-dependent manner, while this drug had no effect on gonococcal adherence (Fig. 4C,D).

4. Discussion

In this study we have investigated the role of VN-binding integrin receptors and PKC in VN-triggered invasion of epithelial cells by Opa_{50}-expressing gonococci. Integrins have previously been implicated in bacterial invasion, the paradigm of which is the zipper-like internalization of *Yersinia* spp. expressing the surface protein invasin, which occurs via high

affinity binding to β_1-integrin receptors [25]. Peptides containing the RGD motif are well characterized to inhibit interaction between RGD containing EM-proteins and the RGD recognizing integrins $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ [17,26]. Among those, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ represent receptors for VN. RGDS peptides have been successfully used to inhibit interaction of $\alpha_v\beta_3$ to VN bound as a bridging molecule to Opc-expressing meningococci [24]. We could demonstrate that the VN triggered entry of *Neisseria gonorrhoeae* into HeLa cells is inhibited by the integrin-blocking RGDS peptide but not by the non-blocking RGES control peptide (Fig. 1), suggesting that this invasion process is mediated by one of the indicated integrin receptors. This hypothesis was further supported by the use of well characterized monoclonal antibodies known to block specifically the interaction of either $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrins with their respective ligands. In addition to blocking integrin-dependent physiological processes like angiogenesis or cell migration [27,28] these antibodies had previously been used to inhibit binding of adenovirus to $\alpha_v\beta_5$ or $\alpha_v\beta_3$ expressed on melanoma cells [21] or bridging of VN bound to Opc-expressing meningococci to $\alpha_v\beta_3$ on endothelial cells [24]. Interesting, we found that blocking of $\alpha_v\beta_5$ by antibody resulted in a nearly complete inhibition of VN-triggered invasion by gonococci, while blocking $\alpha_v\beta_3$ abrogated this invasion process only partly (Fig. 2). Due to a lack of available blocking antibodies for similar studies with other VN-binding integrins, e.g. $\alpha_v\beta_1$ and $\alpha_{IIb}\beta_3$, we cannot exclude that these integrins may also be involved in VN-triggered invasion. However, a prominent role of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins in VN-triggered invasion is further indicated by cell surface receptor density measurements on a panel of epithelial cell lines in which the surface expression of these integrins was found to correlate with the extent of VN-dependent gonococcal invasion (Fig. 3).

Endocytosis of VN has been ascribed to separate pathways which may either depend on $\alpha_v\beta_3$ or $\alpha_v\beta_5$. $\alpha_v\beta_3$-dependent endocytosis further involves a co-ligation of $\alpha_5\beta_1$ [18]. $\alpha_5\beta_1$ was found to be expressed on HeLa cells, however, blocking antibodies did not interfere with VN-dependent invasion (Fig. 2). As no secondary antibody was added, the used monoclonal antibodies should not result in ligation of the integrin as required for promoting $\alpha_v\beta_3$-dependent endocytosis of VN. Together with the only partial inhibition of VN-dependent invasion observed by using blocking antibodies specific for $\alpha_v\beta_3$, the $\alpha_v\beta_3/\alpha_5\beta_1$-dependent pathway for VN-endocytosis does not appear to represent the prominent aspect of the VN-triggered invasion process. Endocytosis of VN via $\alpha_v\beta_5$ was previously shown to depend on an external activation of PKC [29]. By using the serine/threonine kinase inhibitors staurosporine or the PKC specific inhibitor calphostin C we have observed inhibition with a concentration dependency typical

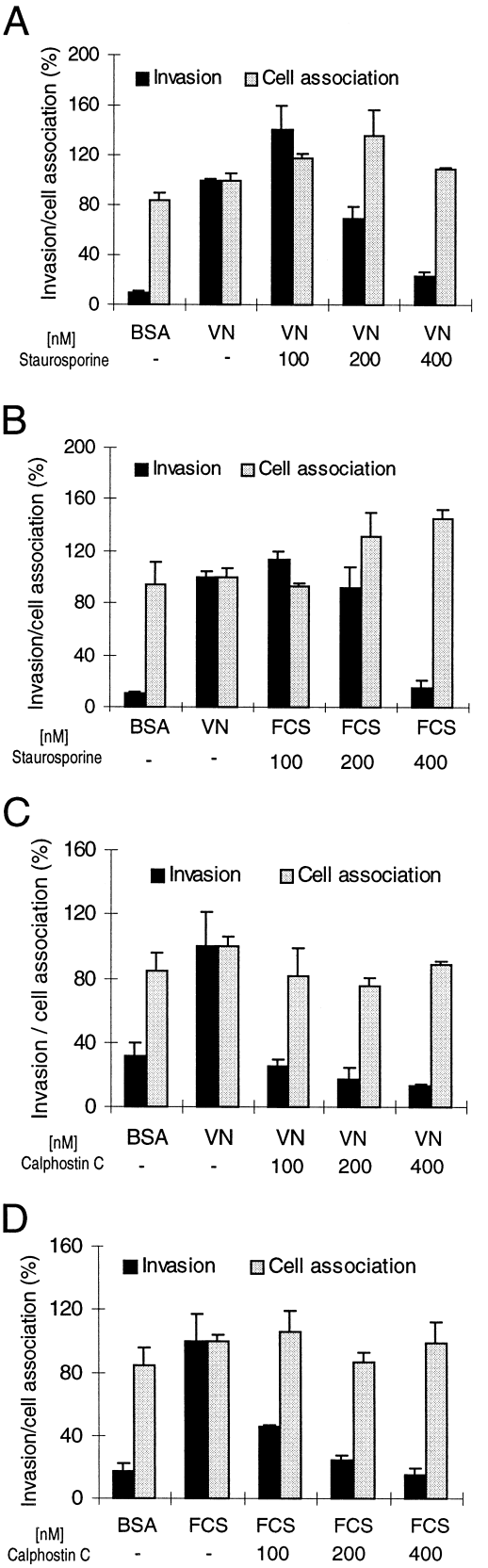

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Fig. 4. Inhibition of VN-triggered invasion of HeLa by Opa_{50}-expressing gonococci in the presence of staurosporine or calphostin C. Bacterial invasion or cell association assays were performed in the presence or absence of (A,C) 2.5 μg/ml human vitronectin (VN) or (B,D) 5% fetal calf serum (FCS) for 6 h. Cells were preincubated for 15 min with (A,B) staurosporine or (C,D) calphostin C at the concentrations indicated prior to the addition of bacteria and the inhibitors were present throughout the infection. The invasion (black bars) or the cell association (gray bars) of (A,C) VN or (B,D) FCS in the absence of further supplements was set to 100% and all other data were expressed in percentages of these reference values. The bars and error bars represent the mean and standard deviation of triplicate samples obtained in one representative experiment.

for inhibition of PKC (Fig. 4). Interestingly, oligomerization of the HSPG syndecan-4 was recently demonstrated to result in the direct binding and activation of PKC [30,31]. Consistent with this, Panetti suggested that the external activation of PKC necessary to mediate $\alpha_v\beta_5$-dependent endocytosis of VN may result from ligation of HSPGs [29]. Based on these reports and the results reported in this study we propose that an efficient gonococcal uptake process into HeLa cells is stimulated by the concerted action of two separate Opa_{50}-mediated receptor interactions: (i) direct binding of Opa_{50} to HSPGs resulting in ligation of these receptors and the subsequent activation of PKC and (ii) the binding of VN to Opa_{50} resulting in the ligation of VN-binding integrins. The co-ligation of HSPGs and of $\alpha_v\beta_5$ may effectively trigger gonococcal uptake by subverting a PKC-dependent process which physiologically serves for endocytosis of VN. As binding of pathogens to either VN and/or HSPGs is a common scheme in microbial pathogenesis, the paradigm of VN-triggered and HSPG-dependent invasion of host cells by gonococci may allow better understanding of some of these still elusive host-pathogen interactions.

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