Pseudomonas aeruginosa Activates PKC-Alpha to Invade Middle Ear Epithelial Cells

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Otitis media (OM) is a group of complex inflammatory disorders affecting the middle ear which can be acute or chronic. Chronic suppurative otitis media (CSOM) is a form of chronic OM characterized by tympanic membrane perforation and discharge. Despite the significant impact of CSOM on human population, it is still an understudied and unexplored research area. CSOM is a leading cause of hearing loss and life-threatening central nervous system complications. Bacterial exposure especially *Pseudomonas aeruginosa* is the most common cause of CSOM. Our previous studies have demonstrated that *P. aeruginosa* invades human middle ear epithelial cells (HMEECs). However, molecular mechanisms leading to bacterial invasion of HMEECs are not known. The aim of this study is to characterize the role of PKC pathway in the ability of *P. aeruginosa* to colonize HMEECs. We observed that otopathogenic *P. aeruginosa* activates the PKC pathway, specifically phosphorylation of PKC-alpha (PKC-α) in HMEECs. The ability of otopathogenic *P. aeruginosa* to phosphorylate PKC-α depends on bacterial OprF expression. The activation of PKC-α was associated with actin condensation. Blocking the PKC pathway attenuated the ability of bacteria to invade HMEECs and subsequent actin condensation. This study, for the first time, demonstrates that the host PKC-α pathway is involved in invasion of HMEECs by *P. aeruginosa* and subsequently to cause OM. Characterizing the role of the host signaling pathway in the pathogenesis of CSOM will provide novel avenues to design effective treatment modalities against the disease.

Keywords: chronic suppurative otitis media, *Pseudomonas aeruginosa*, PKC pathway, PepTag assay, MARCKs

INTRODUCTION

Otitis media (OM) refers to inflammation of the middle ear and mastoid cavity which can be acute or chronic (Minovi and Dazert, 2014; Qureishi et al., 2014). OM accounts for more than 25 million visits to physician's offices annually and is associated with significant healthcare costs (Klein, 2000; Monasta et al., 2012). The global burden of disease study has attributed 4.68 million disability-adjusted life years (DALYs) to OM, a disease burden that is almost as high as the intestinal helminth infections (Murray et al., 2012). OM recurrence rates are high and acute OM (AOM) can progress to chronic OM (COM) despite appropriate treatment (Morris and Leach, 2009). One form of COM

is chronic suppurative otitis media (CSOM) characterized by tympanic membrane perforation and purulent discharge (Verhoeff et al., 2006; Li MG et al., 2015). Children are at greater risk and suffer most frequently from CSOM, causing serious deterioration in their quality of life (Olatoke et al., 2008; Aarhus et al., 2015). CSOM remains an important global public health problem leading to hearing impairment, which may have serious long term effects on language, auditory, and cognitive development, as well as the educational progress of children (Elemraid et al., 2010; Taipale et al., 2011; Kolo et al., 2012; Jensen et al., 2013; Mittal et al., 2015). In addition, due to the proximity of ear to the brain, the spread of suppuration to the central nervous system (CNS) can lead to fatal extracranial and intracranial complications (Seven et al., 2005; Hossain et al., 2006; Dubey et al., 2010; Sun and Sun, 2014). Approximately 28,000 deaths are reported per year from CSOM due to CNS complications including brain abscess and meningitis (Acuin, 2004). Beside advances in medical therapy, CSOM still remains a clinically challenging disease. Despite the significant impact of CSOM on human population, the molecular mechanisms underlying the disease are still unknown. The emergence of antibiotic resistance and potential ototoxicity of antibiotics has created an immediate incentive to develop effective treatment modalities against CSOM. To design these therapeutic strategies, there is a need to understand the pathogenesis of CSOM. Bacterial infection of the middle ear is the most important factor that predisposes individuals to CSOM (Bluestone, 1998). *Pseudomonas aeruginosa* is the most common pathogen associated with CSOM (Saini et al., 2005; Yeo et al., 2007; Dayasena et al., 2011; Madana et al., 2011; Afolabi et al., 2012; Sattar et al., 2012). Our previous studies have demonstrated that *P. aeruginosa* invades human middle ear epithelial cells (HMEECs) and induces cytoskeletal rearrangements (Mittal et al., 2014). However, molecular mechanisms leading to actin condensation and invasion of HMEECs by *P. aeruginosa* are not known.

Protein kinase C (PKC) is a central host molecule that has been implicated in cytoskeletal reorganization (Brandt et al., 2002). A number of actin-binding proteins regulate the structure and dynamics of the actin cytoskeleton through organization of F-actin into a three-dimensional structure (dos Remedios et al., 2003; Paavilainen et al., 2004). Activities of these actin-binding proteins are controlled through various host signaling pathways to ensure proper spatial and temporal regulation of actin dynamics in cells (Khurana and George, 2008). One such signal transduction pathway that affects the actin cytoskeleton is the PKC pathway (Long and Freeley, 2014). PKC regulates the morphology of the F-actin cytoskeleton and thereby influences processes that are affected by remodeling of the microfilaments including cellular migration and neurite growth (Larsson, 2006; Quann et al., 2011; Michalczyk et al., 2013). PKC is composed of a family of phospholipid-dependent serine/threonine kinases mediating diverse cellular responses (Newton, 1995). In general, PKC has a catalytic domain that contains the ATP binding site and a regulatory domain containing the phospholipid and diacylglycerol (DAG) binding site (Luo and Weinstein, 1993; Poli et al., 2014).

Since PKC plays a central role in signaling events leading to changes in the cell membrane and cytoskeleton (Brandt et al., 2002), we hypothesized that PKC activation plays a crucial role in the invasion of HMEECs by *P. aeruginosa*. Our results showed that the PKC pathway is indeed involved in the ability of *P. aeruginosa* to colonize HMEECs and cause actin condensation. PKC inhibitors significantly blocked the invasion of HMEECs by otopathogenic *P. aeruginosa*. We also observed that *P. aeruginosa* of ear origin activates PKC during invasion of HMEECs for which bacterial OprF expression is necessary. The activated PKC translocates to the plasma membrane to initiate downstream signaling transduction events. To the best of our knowledge, this study for the first time demonstrates the role of PKC pathway in the pathogenesis of CSOM.

MATERIALS AND METHODS

Cell Culture

Human middle ear epithelial cells (kindly provided by Dr. David Lim) were generated from human middle ear mucosa as described earlier (Mittal et al., 2014; Woo et al., 2015). HMEECs were cultured and maintained as described earlier (Lim and Moon, 2011; Mittal et al., 2014; Woo et al., 2014, 2015; Val et al., 2015). Briefly, HMEECs were cultured in a 1:1 mixture of Bronchial Epithelial Cell Basal Medium (Lonza, Allendale, NJ, USA) and Dulbecco's Modified Eagle Medium (Cellgro, Manassas, VA, USA) supplemented with bronchial epithelial growth medium (BEGM) Singlequots (Lonza, Allendale, NJ, USA) and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). In some experiments, HMEECs were transfected with DN-PKC-α (Addgene Cambridge, MA, USA; Soh and Weinstein, 2003) using TransIT®-LT1 transfection reagent (Mirus, Madison, WI, USA) as per the manufacturer's instructions. In separate experiments, HMEECs were treated with different concentrations of PKC inhibitors or actin polymerization or microtubule disrupting agents and then subjected to invasion assay.

Bacterial Strains

A clinical otopathogenic strain of *P. aeruginosa* isolated from CSOM patient attending University of Miami Hospital is used in this study. The strain was identified and characterized as described previously (MacFaddin, 1976; Forbes et al., 1998; Saini et al., 2005; Yeo et al., 2007; Dayasena et al., 2011; Madana et al., 2011; Afolabi et al., 2012; Sattar et al., 2012). The isogenic OprF mutant (ΔoprF) and respective plasmid complemented strain (pOprF) consisting of the functional *oprF* gene was generated as described earlier (Woodruff and Hancock, 1989; Horton et al., 1990; Rietsch et al., 2005; Fito-Boncompte et al., 2011; Yakhnina et al., 2015). Bacteria were grown overnight in Luria broth at 37°C in a rotary shaker.

Invasion Assays

Gentamicin protection assays were used to quantify the extent of bacterial invasion of HMEECs (Mittal et al., 2014). Briefly, HMEECs were infected with bacteria at various multiplicity of infection (MOI) and for different time-periods. After incubation,

the cells were washed five times with warm RPMI followed by addition of medium containing gentamicin (200 μg/ml) and further incubated for 1 h at 37°C. The cells were washed three times with RPMI and then lysed with 1% saponin to release intracellular bacteria. Serial dilutions were then plated on blood agar plates and bacterial colonies were counted the next day. The binding of bacteria to HMEECs was determined by lysing the cells without adding gentamicin. To determine the effect of PKC inhibitors, HMEECs were pretreated with different concentrations of PKC inhibitory or control peptide, BIM I, Gö-6976, calphostin C, and chelerythrine for 30 min before infecting with bacteria and maintained in the medium for the entire infection period.

PepTag Assay for Non-radioactive Detection of PKC Activity

The activation of PKC in HMEECs in response to *P. aeruginosa* infection was assessed by PepTag assay as per the manufacturer's instructions (Promega, Madison, WI, USA). The assay uses brightly colored, fluorescent peptide substrates that are highly specific for the kinases in question. The hot pink color is imparted by the addition of a dye molecule to the PepTag Peptide substrate. Phosphorylation of PKC alters the peptide's net charge from +1 to −1. This change in the net charge of the substrate allows the phosphorylated and non-phosphorylated versions of the substrate to be rapidly separated on an agarose gel. The phosphorylated species migrates toward the positive electrode, while the non-phosphorylated substrate migrates toward the negative electrode. HMEECs total cell lysates or membrane fractions (10–25 μg in 10 μl) were incubated with PKC reaction mixture at 33°C for 30 min. The reactions were stopped by placing the tubes in a boiling water bath. Samples were then run on agarose gel and bands were visualized under UV light.

Quantitation of Extent of Phosphorylation by Spectrophotometry

Spectrophotometric method was used to quantitate PKC kinase activity as per manufacturer's instructions. The phosphorylated bands at the negative electrode were excised by scalpel blade immediately after imaging the gel followed by heating at 95°C. The volume of the solution was adjusted to 250 μl with water. Samples were then mixed with gel solubilization buffer and glacial acetic acid followed by transferring to 96 well plates and reading the absorbance at 570 nm. Results were expressed in kinase units/ml.

Western Blotting and Immunoprecipitation

Cells were infected with bacteria for different post-infection time periods, and lysates prepared. Protein concentrations of the extracts were measured using a BCA assay (Pierce, Rockford, IL, USA). Equivalent amounts of extracts were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (Mittal and Prasadarao, 2010). For quantification, the Densitometric analysis was done using

ImageJ software. For immunoprecipitation, cell lysates were incubated with anti-actin antibody overnight at 4°C followed by addition of Protein A+G sepharose beads. Immunoprecipitated beads were washed, resuspended in SDS sample buffer and resolved on SDS-PAGE. Immunoprecipitated proteins were transferred to nitrocellulose membrane and analyzed by Western blotting with anti- phospho-PKC-α antibody.

Preparation of Membrane and Cytosolic Fractions

The membrane and cytosolic fractions were prepared from HMEECs using commercially available kit (Biovision, Milpitas, CA, USA) as per the manufacturer's instructions.

Immunofluorescence Microscopy

For staining of bacteria and actin, HMEECs were cultured in 8-well chamber slides and infected with *P. aeruginosa* for varying time periods. After incubation, cells were washed three times with PBS buffer and then fixed and permeabilized with BD cytofix and cytoperm reagent (BD Biosciences, San Jose, CA, USA) for 30 min. After washing, the cells were blocked with 3% normal goat serum (NGS) for 30 min and then incubated with anti-Phospho PKC-α antibody (Abcam, Cambridge, MA, USA) for 45 min followed by Alexa Fluor 488 antibody (Life Technologies, Carlsbad, CA, USA). After washing, cells were counterstained for actin with rhodamine phalloidin (Life Technologies, Carlsbad, CA, USA) for 45 min, washed and mounted in an antifade Vectashield solution containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The cells were viewed with a Zeiss LSM 710 microscope (Carl Zeiss, Germany) and images were assembled using Adobe Photoshop 7.0.

Statistical Analysis

Statistical significance was determined by a paired, two-tailed Student's *t*-test using SPSS software. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

PKC Inhibitors Attenuate Invasion of HMEECs by *P. aeruginosa*

Our previous studies have demonstrated that *P. aeruginosa* can invade HMEECs and induce cytopathic effects (Mittal et al., 2014). To determine the role of PKC in cell invasion, HMEECs were infected with *P. aeruginosa* in the presence and absence of different concentrations of PKC inhibitors, bisindolylmaleimide I (BIM I), Gö-6976, PKC inhibitory peptide, calphostin C and chelerythrine. Bacterial cell invasion was then determined by gentamicin protection assay. We observed that with increase in concentrations of PKC inhibitors, there was significant decrease in invasion of HMEECs by *P. aeruginosa* ($P < 0.01$; **Figures 1A–C**). A concentration of 10 μM of BIM I was able to inhibit the invasion of *P. aeruginosa* by 50% whereas a dose of
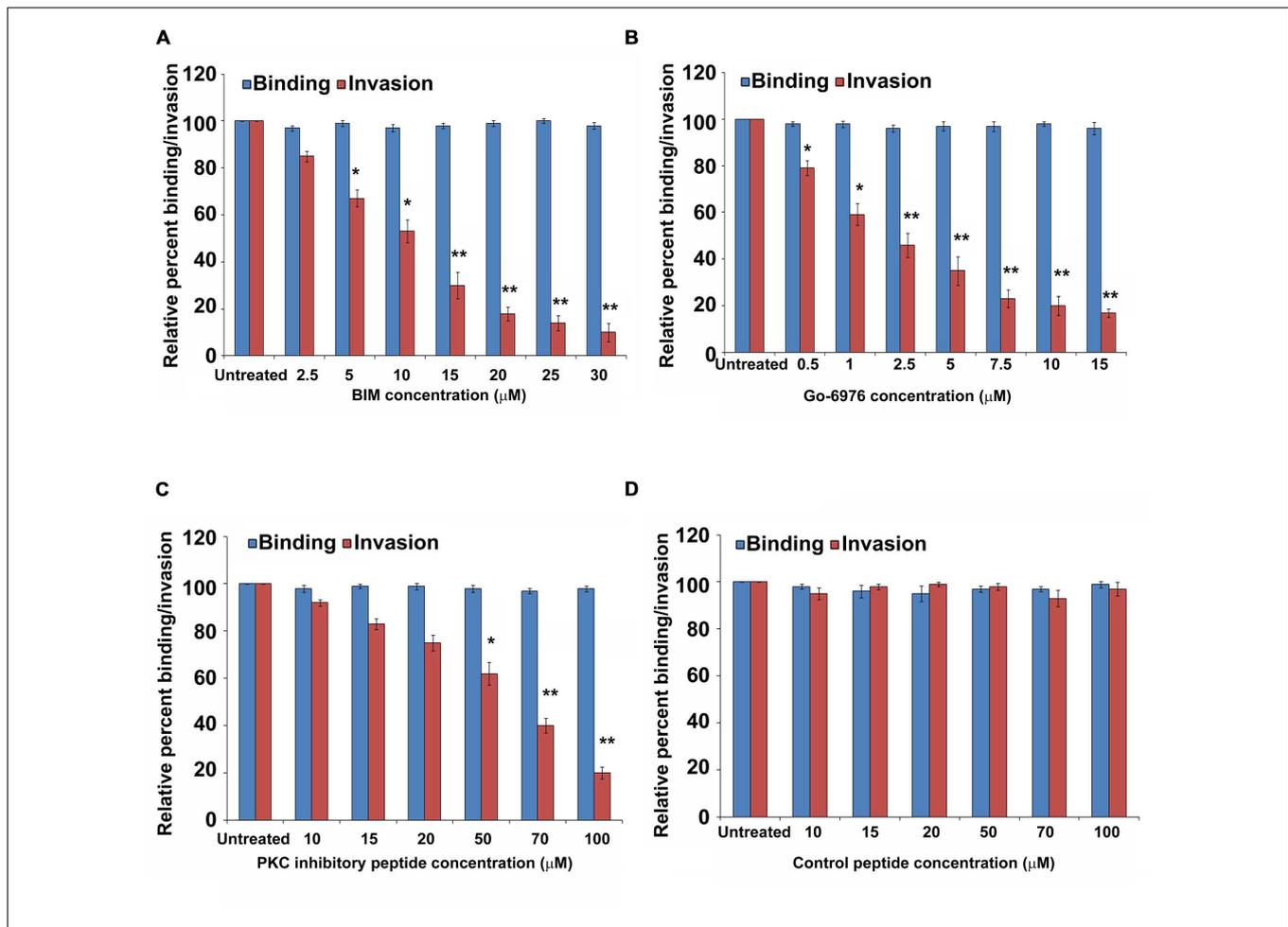
FIGURE 1 | PKC inhibitors prevent invasion of HMEECs by *P. aeruginosa*. HMEECs were pretreated with different concentrations of BIM I **(A)**, Gö-6976 **(B)**, PKC inhibitory peptide **(C)**, or control peptide **(D)** for 30 min and then infected with *P. aeruginosa* at an MOI of 10 for 2 h. Bacterial adhesion and invasion was then determined. The results are expressed as percentage compared to the bacterial adhesion/invasion in untreated infected cells. Data represents mean ± SD and is representative of four individual experiments carried out in triplicate. *$P < 0.01$ or **$P < 0.001$ compared to control.

30 μM was able to inhibit invasion by 90% (**Figure 1A**). Gö-6976 was able to block the invasion of *P. aeruginosa* by 85% at a concentration of 15 μM (**Figure 1B**). The myristoylated PKC inhibitory peptide at a concentration of 50 μM was able to block the invasion by 40% whereas a dose of 90 μM was able to inhibit the cell invasion by 80% (**Figure 1C**). However, control peptide had no effect on the invasion of *P. aeruginosa* into HMEECs at any of the tested concentrations (**Figure 1D**). Similar results of decreased cell invasion was observed with other PKC inhibitors, calphostin C and chelerythrine (Supplementary Figures S1 and S2). We did not observe any difference in the binding of *P. aeruginosa* to HMEECs in the presence of PKC inhibitors (**Figures 1A–C** and Supplementary Figures S1 and S2) indicating that the observed differences in invasion were not due to the differential bacterial binding. We also did not observe any toxic effects of these reagents on bacteria or on cells at the tested concentrations (data not shown). These results suggest that PKC plays a crucial role in colonization of HMEECs by *P. aeruginosa*.

P. aeruginosa Activates PKC during HMEECs Invasion for Which OprF Expression is Required

Since we observed that PKC plays a role in cell invasion, next we determined whether *P. aeruginosa* activates PKC in HMEECs. Our previous studies have demonstrated that bacterial OprF expression is required for the invasion of HMEECs by *P. aeruginosa* (Mittal et al., 2014). Therefore, we examined the ability of wild-type (WT) and Δ*oprF* mutant of *P. aeruginosa* to activate PKC in HMEECs by PepTag assay. Cells were infected with otopathogenic *P. aeruginosa* for varying time periods and PKC activation was determined using non-radioactive PepTag assay. HMEECs infected with WT *P. aeruginosa* demonstrated PKC activation within 30 min, which peaked at 90 min and then decreased at 120 min post-infection (**Figure 2A**). The increased PKC activity was approximately fourfold compared with PKC activity in control uninfected cells as estimated by the spectrophotometric method using phosphorylated substrate
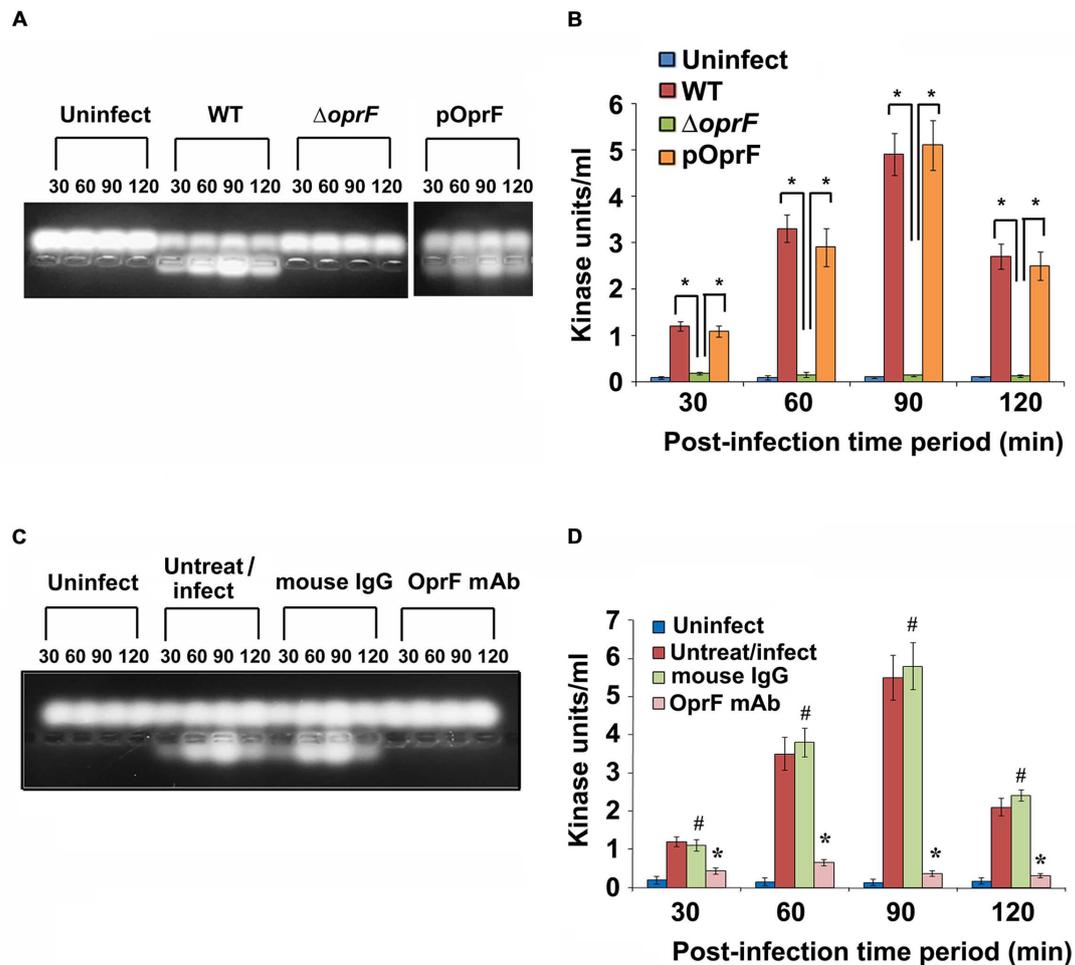
FIGURE 2 | Otopathogenic _P. aeruginosa_ requires OprF to activate PKC in HMEECs. Cells were infected with different strains of _P. aeruginosa_ for varying time periods and subjected to non-radioactive PepTag assay **(A)**. PKC activity was quantified by excising the phosphorylated bands from the agarose gel and results were expressed in kinase units/ml **(B)**. In separate experiments, _P. aeruginosa_ was pretreated with anti-OprF monoclonal antibody (mAb) or mouse IgG or left untreated and then used in the PKC assay **(C,D)**. Data represents mean ± SD. Results are representative of five independent experiments. $^{\#}P > 0.05$ or $^{*}P < 0.001$.

peptides (**Figure 2B**). In contrast, $\Delta oprF$ mutant induced significantly lower PKC activation than WT _P. aeruginosa_ ($^{*}P < 0.01$). However, the cells infected with the complemented strain (pOprF strain) showed a similar pattern of PKC activation as the WT strain. These results suggest that interaction of OprF with HMEECs activates downstream signaling leading to cell invasion by _P. aeruginosa_.

To determine whether OprF has direct influence on the ability of _P. aeruginosa_ to activate PKC, bacteria were pretreated with anti-OprF monoclonal antibody (mAb) and then used to infect HMEECs. Bacteria pretreated with isotype antibody or left untreated and uninfected served as the control group. We observed that anti-OprF monoclonal antibody treated bacteria failed to elicit PKC activation in HMEECs (**Figures 2C,D**). These results suggest that OprF directly contributes to the activation of PKC in HMEECs in response to _P. aeruginosa_ infection.

EGTA Treatment Abrogates _P. aeruginosa_ Induced PKC Activation

Calcium plays an important role in the activation and regulation of conventional PKC isoforms which are most commonly implicated in cytoskeletal rearrangements (Herbert et al., 1990; Farah and Sossin, 2012). Therefore, we treated HMEECs with ethylene glycol tetraacetic acid (EGTA) to chelate calcium and infected with _P. aeruginosa_. Total cell lysates were then prepared and subjected to PepTag assay. We observed that PKC activation was completely abolished following pretreatment with EGTA (**Figure 3A**). Quantification of this data confirmed that EGTA significantly abrogated _P. aeruginosa_ induced PKC activation in HMEECs compared to untreated cells ($^{*}P < 0.001$; **Figure 3B**). These findings suggest that the PKC activated in HMEECs following _P. aeruginosa_ can be the conventional PKC isoform, PKC-α.
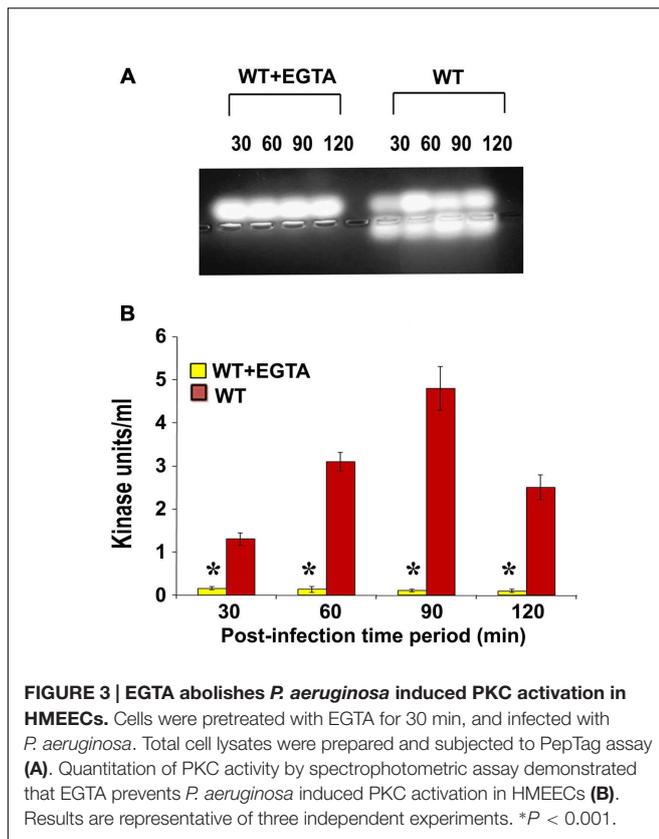
FIGURE 3 | EGTA abolishes *P. aeruginosa* induced PKC activation in **HMEECs.** Cells were pretreated with EGTA for 30 min, and infected with *P. aeruginosa*. Total cell lysates were prepared and subjected to PepTag assay **(A)**. Quantitation of PKC activity by spectrophotometric assay demonstrated that EGTA prevents *P. aeruginosa* induced PKC activation in HMEECs **(B)**. Results are representative of three independent experiments. *$P < 0.001$.

Otopathogenic *P. aeruginosa* Specifically Activates PKC-α in HMEECs

Next we set forth to determine the PKC isotype activated by *P. aeruginosa* in HMEECs. PKC family is comprised of different isoforms that trigger distinct host signal transduction pathways (Newton, 1995). Cells were infected with WT, Δ*oprF* mutant or pOprF strains of *P. aeruginosa* for varying time-period, total cell lysates prepared and subjected to Western blotting. We observed that *P. aeruginosa* activated only PKC-α but not the other isoforms namely, PKC-γ and PKC-δ (**Figure 4A**). Time course study using anti-phospho-PKC-α specific antibodies revealed that *P. aeruginosa* phosphorylates PKC-α within 30 min post-infection, showing a peak at 90 min post-infection, and then decreases gradually afterward (**Figure 4B**). However, Δ*oprF* mutant failed to show any phosphorylation of PKC-α (**Figure 4A**). The ability of Δ*oprF* mutant to phosphorylate PKC was regained following complementation with pOprF. This data confirms our earlier PepTag assay data that phosphorylation of PKC-α in response to *P. aeruginosa* requires bacterial OprF expression.

Pseudomonas aeruginosa Induces Translocation of PKC to the Membrane

The migration of phosphorylated PKC to the plasma membrane is essential in order to initiate downstream signaling (Newton, 1995). Therefore, we examined whether phosphorylated PKC in response to *P. aeruginosa* infection translocate to the
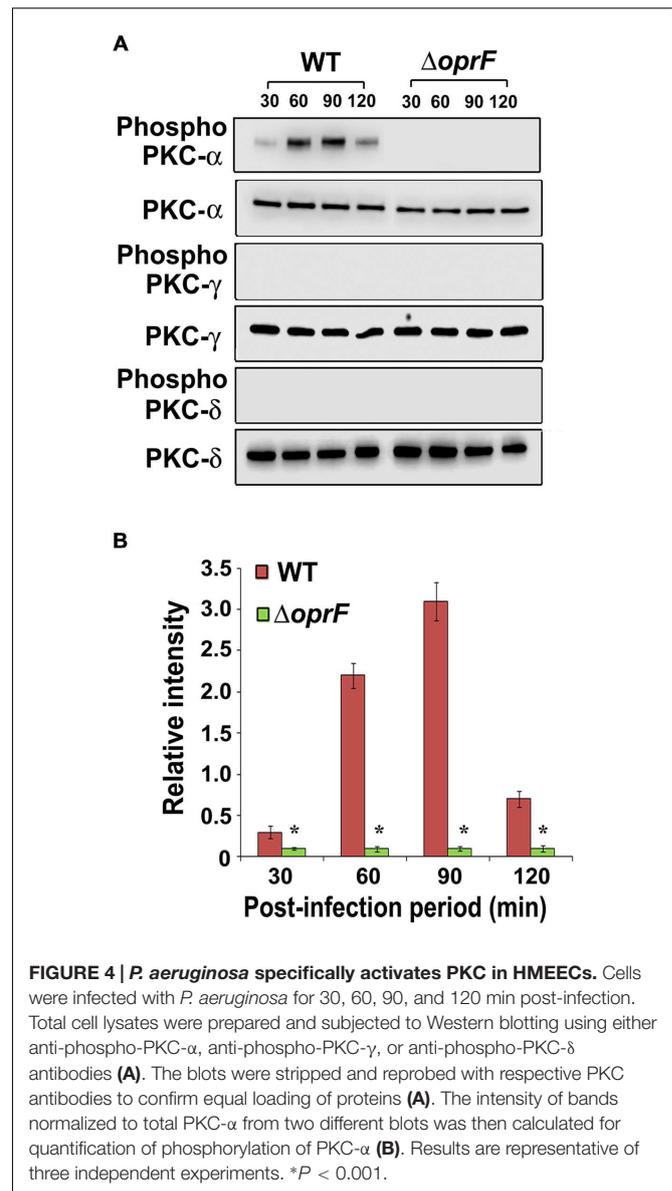
FIGURE 4 | *P. aeruginosa* specifically activates PKC in HMEECs. Cells were infected with *P. aeruginosa* for 30, 60, 90, and 120 min post-infection. Total cell lysates were prepared and subjected to Western blotting using either anti-phospho-PKC-α, anti-phospho-PKC-γ, or anti-phospho-PKC-δ antibodies **(A)**. The blots were stripped and reprobed with respective PKC antibodies to confirm equal loading of proteins **(A)**. The intensity of bands normalized to total PKC-α from two different blots was then calculated for quantification of phosphorylation of PKC-α **(B)**. Results are representative of three independent experiments. *$P < 0.001$.

plasma membrane of HMEECs. HMEECs were infected with *P. aeruginosa* and membrane fractions were prepared using commercially available kit (Biovision, Milpitas, CA, USA). PepTag assay revealed that phosphorylated PKC resides in the membrane (**Figure 5A**). We observed the same pattern of PKC activation in membrane fractions as we observed in total cell lysates. There was a fourfold increase in PKC activity at 90 min post-infection as compared to that at 30 min post-infection followed by a gradual decline in PKC activity at 120 min post-infection (**Figure 5B**).

To further confirm these results, we subjected the membrane fractions to Western blotting. We observed that in agreement with our PepTag assay, phosphorylated PKC-α was located in the membrane fraction (**Figure 5C**). There was an increase in phosphorylation of PKC-α with a corresponding increase in post-infection time period from 30 to 90 min as indicated by increase
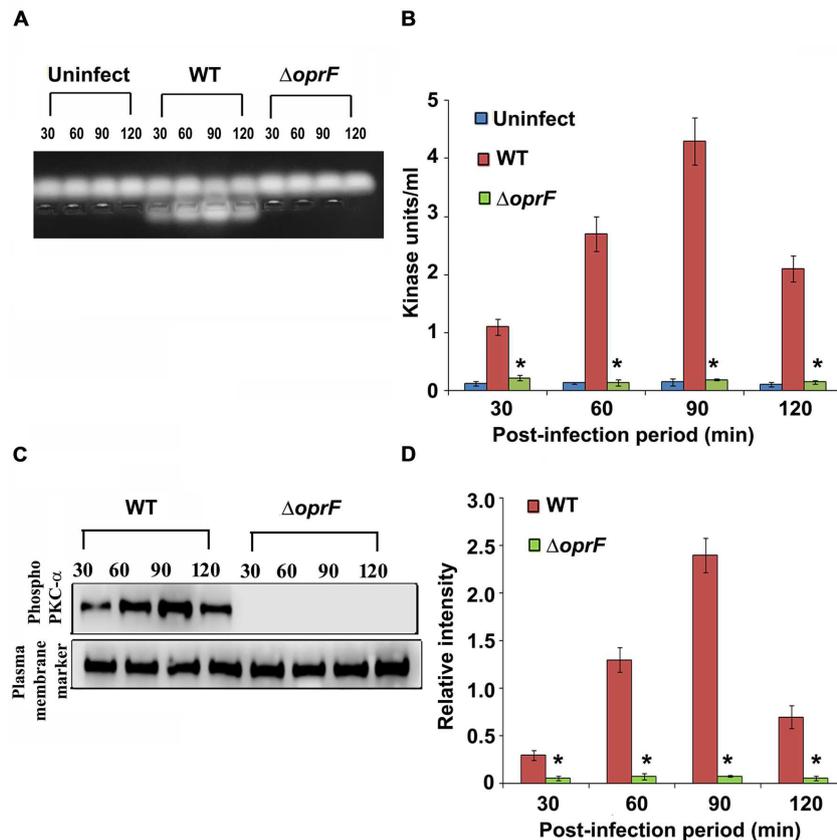
FIGURE 5 | Activated PKC localizes in the plasma membrane. To determine the localization of activated PKC, membrane fractions were prepared from *P. aeruginosa* infected HMEECs and subjected to PepTag assay **(A)**. Quantitation of data showed that activated PKC resides in the membrane fraction **(B)**. In separate experiments, membrane fractions prepared from *P. aeruginosa* infected HMEECs were subjected to Western blotting and probed with anti-phospho-PKC-α antibody **(C)**. The blots were stripped and reprobed with anti-alpha 1 sodium potassium ATPase antibody (plasma membrane marker) to confirm equal loading of proteins. The intensity of bands normalized to plasma membrane marker was then calculated for quantification from two different blots **(D)**. Results are representative of three independent experiments. *$P < 0.001$.

in band intensity followed by a gradual decline at 120 min post-infection (**Figure 5D**). Taken together, these results suggest that *P. aeruginosa* induced activated PKC translocate to the plasma membrane.
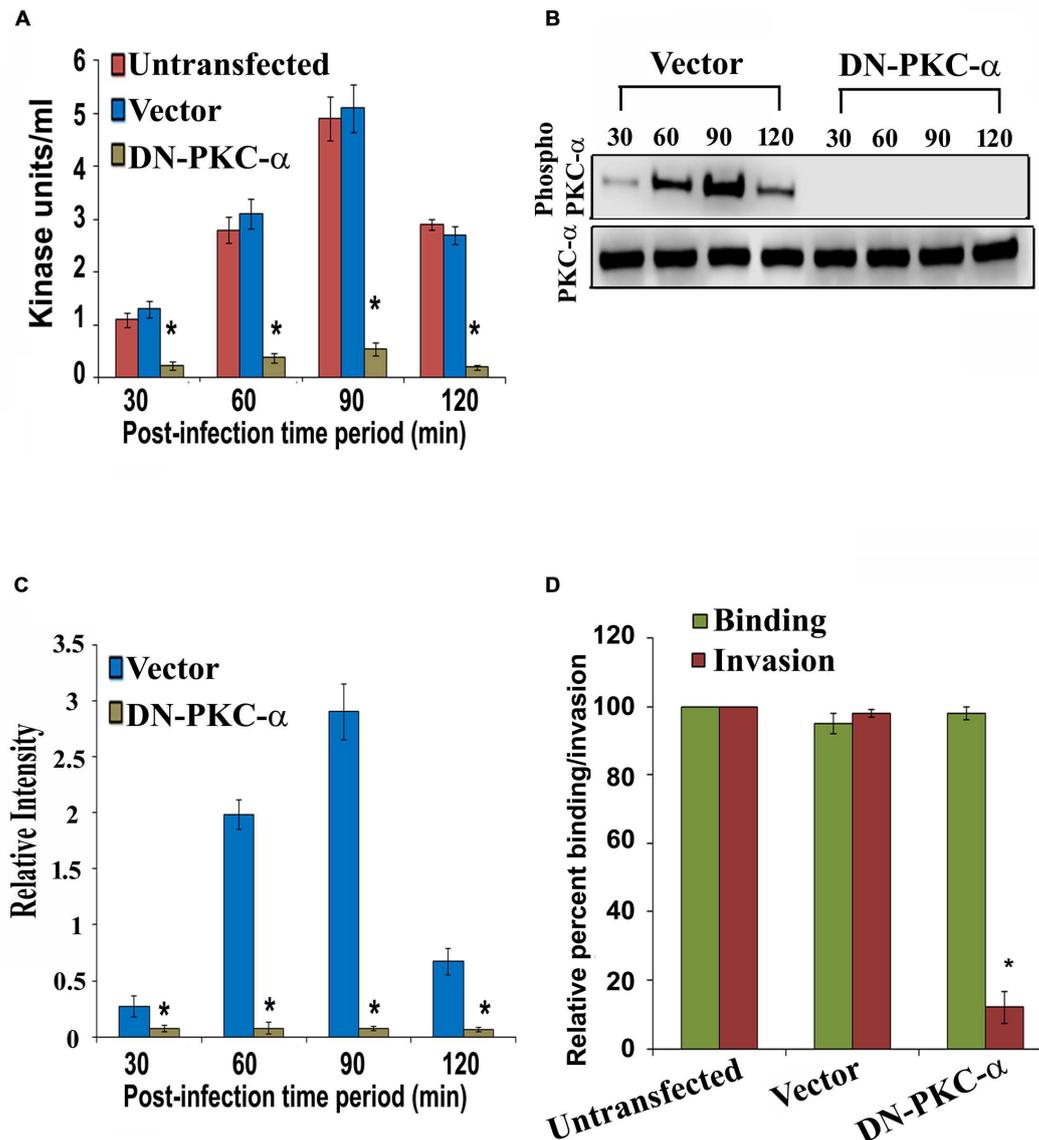
Overexpression of Dominant Negative Form of PKC-α Inhibits PKC Activation and Invasion of HMEECs by *P. aeruginosa*

To characterize the role of PKC-α in cell invasion by *P. aeruginosa*, HMEECs were transfected with dominant negative (DN) PKC-α plasmid or vector alone or left untransfected. The DN PKC-α expression plasmid (PKC-α-cat/KR) encodes a truncated protein in which the N-terminal regulatory domain is deleted, while the catalytic domain (cat) containing amino acids 326–672 of PKC is preserved with a point mutation that abolishes the ATP binding ability (Soh and Weinstein, 2003). To determine whether expression of DN-PKC-α will prevent the activation of *P. aeruginosa* induced PKC, HMEECs lysates were

subjected to PepTag assay. We observed that PKC activation was significantly attenuated in DN-PKC-α transfected cells compared to vector control or untransfected cells (**Figure 6A**). DN-PKC-α transfected cells showed fivefold decrease in PKC activation compared to vector control or untransfected cells ($P < 0.001$; **Figure 6A**). Similar results of decreased expression of PKC-α in DN-PKC-α transfected cells were observed with Western blotting (**Figures 6B,C**). In agreement with this data, the expression of DN-PKC-α into HMEECs significantly prevented the invasion of *P. aeruginosa* into HMEECs (**Figure 6D**). However, we did not observe any significant decrease in adhesion of *P. aeruginosa* to HMEECs (**Figure 6D**). This data demonstrates that colonization of HMEECs by *P. aeruginosa* requires active PKC-α.

Phospho PKC-α Associates with Actin in *P. aeruginosa* Infected HMEECs

In our previous studies, we observed that *P. aeruginosa* induces actin condensation during cell invasion (Mittal et al., 2014). Since PKC can play a central role in cytoskeletal rearrangements,
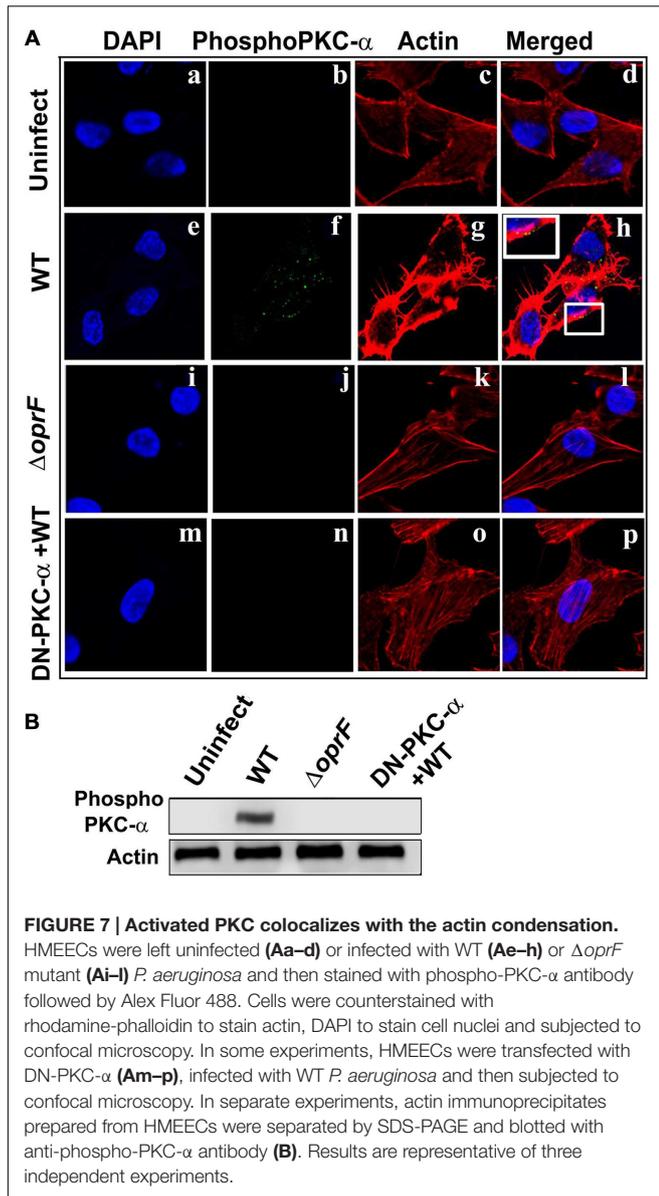
FIGURE 6 | Overexpression of dominant-negative (DN) PKC-α abolishes PKC activation and *P. aeruginosa* invasion. HMEECs were transfected with DN PKC-α or vector alone or left untransfected, infected with *P. aeruginosa* and then subjected to PepTag assay. PKC kinase activity was then determined in DN PKC-α transfected cells or vector alone or untransfected HMEECs **(A)**. In separate experiments, total cell lysates from *P. aeruginosa* infected DN PKC-α or vector alone or left untransfected were subjected to Western blotting **(B)**. The extent of PKC-α phosphorylation was determined by densitometric analysis normalized to PKC-α from two different blots **(C)**. The invasion and adhesion of *P. aeruginosa* was also determined **(D)**. Results are representative of four independent experiments carried out in triplicate. *$P < 0.001$.

we determined whether phospho-PKC-α colocalizes with actin bundles in *P. aeruginosa* infected HMEECs. Cells were infected with *P. aeruginosa* and stained with phospho-PKC-α antibody followed by secondary staining with Alexa Fluor 488. Cells were counterstained with rhodamine phalloidin to stain actin. Uninfected cells showed long actin filaments and no phospho-PKC-α staining (**Figures 7Aa–d**). In contrast, *P. aeruginosa* infected cells demonstrated actin accumulation beneath the bacterial binding sites that co-localizes with phospho-PKC-α staining at majority of sites as indicated by yellow color

(**Figures 7Ae–h**). However, HMEECs infected with Δ*oprF* mutant failed to show actin condensation and phospho-PKC-α staining (**Figures 7Ai–l**). The overexpression of DN-PKC-α also prevented actin condensation and phosphorylation of PKC in agreement with our earlier data (**Figures 7Am–p**).

To confirm whether phospho-PKC-α associates with actin, we immunoprecipitated actin and then probed for phospho-PKC-α in *P. aeruginosa* infected HMEEC lysates. On par with our confocal microscopy results, we observed that phosphorylated PKC-α associates with actin in *P. aeruginosa* infected HMEECs

FIGURE 7 | Activated PKC colocalizes with the actin condensation.
HMEECs were left uninfected **(Aa–d)** or infected with WT **(Ae–h)** or Δ*oprF*
mutant **(Ai–l)** *P. aeruginosa* and then stained with phospho-PKC-α antibody
followed by Alex Fluor 488. Cells were counterstained with
rhodamine-phalloidin to stain actin, DAPI to stain cell nuclei and subjected to
confocal microscopy. In some experiments, HMEECs were transfected with
DN-PKC-α **(Am–p)**, infected with WT *P. aeruginosa* and then subjected to
confocal microscopy. In separate experiments, actin immunoprecipitates
prepared from HMEECs were separated by SDS-PAGE and blotted with
anti-phospho-PKC-α antibody **(B)**. Results are representative of three
independent experiments.

(**Figure 7B**). However, no such association between actin
and phospho-PKC-α was observed in Δ*oprF* mutant infected
HMEECs or cells overexpressing DN-PKC-α. These results
suggest that activated PKC-α in *P. aeruginosa* infected HMEECs
interacts with actin cytoskeleton.

Infection of HMEECs with *P. aeruginosa* Promotes Translocation of MARCKs from Plasma Membrane to Cell Cytosol
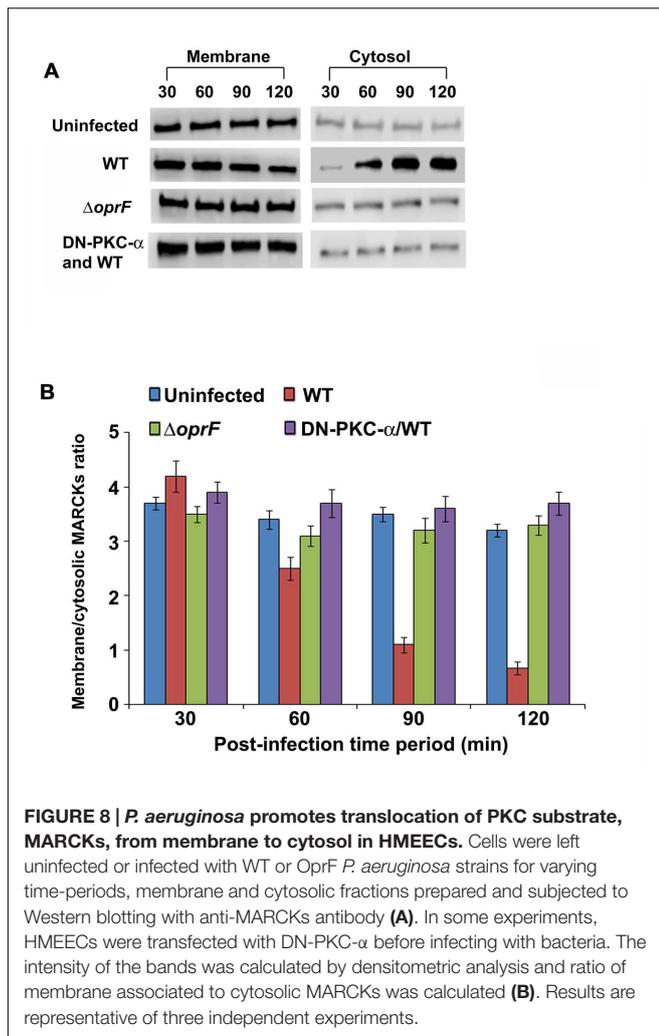
The myristoylated alanine-rich C kinase substrate (MARCKS)
is one of the prominent substrates of PKC (Arbuzova et al.,
2002). MARCKS normally resides in the plasma membrane
where it plays a crucial role in the regulation of actin cytoskeleton
(Arbuzova et al., 2002). Upon activation by PKC, MARCKS
migrates from plasma membrane to the cytosol. Therefore we

examined whether MARCKS translocate from plasma membrane
to cytosol in HMEECs in response to *P. aeruginosa* infection.
HMEECs were infected with *P. aeruginosa*, cytosolic and
membrane fractions prepared and subjected to Western blotting.
We observed that there was a decrease in MARCKs band
intensity with increase in time period from 30 to 120 min
post-infection in the membrane fraction of HMEECs infected
with WT *P. aeruginosa* (**Figure 8A**). Quantification of this data
confirmed that there was a 3.5-fold decrease in the presence
of MARCKs in the membrane fraction at 120 min post-
infection compared to at 30 min post-infection. On the other
hand, MARCKS band intensity increased with increase in post-
infection time period in the cytosolic fraction (**Figure 8A**).
Densitometric analysis revealed a fivefold increase in MARCKs
expression in the cytosol at 120 min post-infection compared
to at 30 min post-infection. On the contrary, Δ*oprF* mutant
showed no increase in the MARCKs expression in the cytosol or
its decrease in the membrane fraction (**Figures 8A,B**). This data
suggest that OprF expression on *P. aeruginosa* is involved in the
translocation of MARCKs from membrane to cytosol. However,
the expression of DN-PKC-α prevented the translocation of
MARCKs from membrane to cytosol in WT *P. aeruginosa*
infected cells suggesting that activation of PKC is necessary for
this translocation (**Figures 8A,B**).

DISCUSSION

Chronic suppurative otitis media is the most prevalent disease
worldwide, especially in developing countries, associated with
potentially serious long term sequelae including hearing loss
and fatal brain diseases. CSOM refers to insidious and chronic
intractable inflammation of mucosa as well as submucosa with
destruction of bone of the middle ear cleft characterized by
persistent perforation of the tympanic membrane and recurrent
otorrhea. The presence of bacteria in the middle ear and mastoid
cavity is the most common cause of CSOM. However, the
molecular mechanisms underlying CSOM are not known.

The invasion of bacteria into the host cells is considered
a prerequisite to cause infection. Our previous studies have
demonstrated that *P. aeruginosa* invades HMEECs in a dose and
time dependent manner. However, molecular mechanisms that
leads to colonization of HMEECs are not known. In this study, we
demonstrated that PKC plays a central role in HMEECs invasion
by *P. aeruginosa*. PKC specific inhibitors such as BIM I, Gö-
6976, PKC inhibitory peptide, calphostin C and chelerythrine
were able to block the bacterial cell invasion by more than
70–80%. BIM I is a cell permeable, very potent, specific, and
selective inhibitor of PKC (Wanger et al., 2015). Gö-6976 is
another PKC inhibitor that prevents the activation of calcium
dependent isoforms in nanomolar concentrations while having
no effect on the kinase activity of the calcium-independent PKC
subtypes even at micromolar doses (Wang et al., 2015). PKC
inhibitory peptide resembles the pseudosubstrate sequence of
PKC-α whose function is to keep these kinases in their inactive
state; thus, this peptide is a very specific competitive inhibitor of
PKC-α (Eichholtz et al., 1993). Myristoylation of PKC inhibitory

FIGURE 8 | *P. aeruginosa* promotes translocation of PKC substrate, MARCKs, from membrane to cytosol in HMEECs. Cells were left uninfected or infected with WT or OprF *P. aeruginosa* strains for varying time-periods, membrane and cytosolic fractions prepared and subjected to Western blotting with anti-MARCKs antibody **(A)**. In some experiments, HMEECs were transfected with DN-PKC-α before infecting with bacteria. The intensity of the bands was calculated by densitometric analysis and ratio of membrane associated to cytosolic MARCKs was calculated **(B)**. Results are representative of three independent experiments.

peptide makes it cell permeable and facilitates the entry inside cells. Calphostin C is a perylenequinone metabolite that targets classical and novel PKC isoforms and inhibits both phorbol ester binding and phosphotransferase activity of PKC through binding to the regulatory domain (Kobayashi et al., 1989; Xiao et al., 2015). Chelerythrine is a benzophenanthridine that is a potent and selective antagonist of classical as well as novel PKCs that targets the catalytic domain (Li SJ et al., 2015). Our results are in agreement with the findings of previous studies that showed that PKC inhibitors can prevent cell invasion by pathogenic microbes. The entry of a number of pathogenic viruses into host cells including the vesicular stomatitis virus, herpes simplex I virus, turkey herpes virus, vaccinia virus, Sindbis virus, human herpesvirus 8, and adenovirus type 2 has been shown to be blocked by PKC inhibitors (Constantinescu et al., 1991; Naranatt et al., 2003; Sieczkarski et al., 2003). BIM-I inhibits the entry of influenza virus inside Mink lung epithelial cells (Mv-1) during early infectious stages without affecting the viral binding as observed in the present study (Root et al., 2000). Earlier studies have also demonstrated that *Cryptosporidium* species utilizes PKC pathway to invade primary human and bovine intestinal

cells (Hashim et al., 2006). PKC inhibitors significantly prevented the cell invasion by *Cryptosporidium* species. In agreement with our invasion data, we observed that *P. aeruginosa* activates PKC in HMEECs as early as 30 min post-infection, showing peak activation at 90 min post-infection. However, to the best of our knowledge, this study for the first time demonstrated the PKC activation for the invasion of HMEECs by otopathogenic bacteria.

PKCs are involved in a large variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation, and cell death (Newton, 1995). At least twelve different isoforms of PKC have been reported which have been categorized into three main types based on calcium dependency and activators: classical or conventional PKCs that are calcium dependent and DAG sensitive namely PKC-α, βI, βII, and γ; novel PKCs (δ, η, θ, μ, ε, ξ) activated by DAG but not calcium; and atypical PKCs (μ, ζ, υ, ι) that are calcium independent and DAG insensitive (Newton, 1995). Each isotype triggers a different downstream signaling pathway that can determine the ultimate outcome of an infection. Therefore, there is a need to decipher the role of PKC during CSOM in order to understand the pathogenesis of the disease. In our study, we observed that PKC activation in HMEECs in response to *P. aeruginosa* is abrogated in the presence of calcium chelator, EGTA. This suggests that *P. aeruginosa* activates the calcium dependent classical isoform of PKC, most probably PKC-α. In agreement with this data, expression of DN PKC-α significantly prevented the invasion of HMEECs by otopathogenic *P. aeruginosa* and subsequent PKC activation. Western blotting demonstrated that otopathogenic *P. aeruginosa* specifically activates PKC-α but not the other isotypes. Interestingly, PKC-α activation has been correlated with increased cytokine production including TNF-α and IL-1β (Redig and Platanias, 2007). Therefore, it is possible that activation of PKC-α lead to exaggerated production of cytokines that is an important hallmark of CSOM. High levels of cytokines leads to tissue damage that further exacerbates the infection and worsens the complications associated with CSOM.

Outer membrane proteins (OMPs) play an important role in the interaction of pathogens with host cells (Galdiero et al., 2012; Confer and Ayalew, 2013). In this study, we observed that ability of otopathogenic *P. aeruginosa* to phosphorylate PKC-α depends on OprF expression. OprF is one of the most abundant OMPs/porin of *P. aeruginosa* (Sugawara et al., 2012). It has been demonstrated that OprF controls the production of the quorum-sensing-dependent virulence factors pyocyanin, elastase, lectin PA-1L, and exotoxin A as well as Type III secretion system associated enzymes, ExoS and ExoT, in non-otopathogenic strains of *P. aeruginosa* (Fito-Boncompte et al., 2011). However, our previous studies have demonstrated that OprF plays a direct role in interaction of otopathogenic *P. aeruginosa* with HMEECs (Mittal et al., 2014). The pretreatment of HMEECs with exogenous OprF or pretreatment of WT *P. aeruginosa* with anti-OprF mAb significantly reduced bacterial cell invasion. On par with these findings, in this study also, we observed that pretreatment of WT bacteria with OprF antibody abrogates *P. aeruginosa* induced PKC-α activation in HMEECs. These

results suggest that OprF plays a unique role in otopathogenic *P. aeruginosa* and is directly involved in bacterial interaction with HMEECs.

The migration of activated PKC to the cell plasma membrane is essential to initiate the subsequent downstream signaling. In most cell types, PKCs are present in a primed, yet inactive, conformation in the cytosol, which are translocated to the membrane upon activation. PKC attains its active conformation when DAG recruits the inactive PKC to the membrane in a calcium-dependent manner, where membrane binding provides sufficient energy to disengage the pseudosubstrate, thus exposing the substrate binding site. We observed that activated PKC in response to *P. aeruginosa* migrates to the cell membrane. It is possible that translocation of phosphorylated PKC-α to the cell membrane facilitates the entry of *P. aeruginosa* inside HMEECs. A significant decrease in invasion of HMEECs by *P. aeruginosa* in the presence of PKC inhibitory peptide, which prevents the translocation of PKC to the membrane, further demonstrates the requirement of membrane-associated active PKC for the successful bacterial cell invasion. These results are consistent with the findings of other studies where activated PKC has been shown to translocate to the plasma membrane. The adhesion of *Leishmania donovani* to macrophages has been shown to produce rapid and transient redistribution of PKC from the cytosol to the plasma membrane (Bhunia et al., 1996). Enteropathogenic *E. coli* has also been shown to activate PKC in HeLa and T84 cells which then migrate to the plasma membrane (Crane and Oh, 1997).

Cytoskeletal rearrangements play an important role in entry and invasion of host cells by pathogens (Truong et al., 2014). In our earlier study, we showed that *P. aeruginosa* induces actin condensation during invasion of HMEECs. Here, we observed that *P. aeruginosa* utilizes PKC pathway to cause these cytoskeletal rearrangements. Confocal microscopy revealed that activated PKC-α colocalizes with the actin beneath the bacterial binding sites. In agreement with this findings, we observed the translocation of MARCKS from membrane to cytosol in *P. aeruginosa* infected HMEECs. MARCKs is a major PKC substrate that is associated with the plasma membrane and promotes cross linking of F-actin filaments in the dephosphorylated state (Blackshear, 1993). Phosphorylation of MARCKS by PKC facilitate its translocation from the plasma membrane to the cytosol leading to actin polymerization

(Aderem, 1995). Thus, the translocation of MARCKs from membrane to cytosol leads to actin cytoskeletal rearrangement that facilitates the entry of *P. aeruginosa* into HMEECs.

In summary, our study provides novel insights into the pathogenesis of CSOM and decipher the role of PKC signaling pathway in the ability of *P. aeruginosa* to cause chronic ear infection. A comprehensive understanding of the role of PKC-α during CSOM employing animal models will pave the way to design effective treatment modalities against the disease and prevent consequent hearing loss as well as life-threatening CNS disorders.

AUTHOR CONTRIBUTIONS

RM, MG, DY, and XL conceived and designed the study. RM and MG performed the experiments and analyzed the data. RM, MG, DY, and XL wrote the manuscript. All the authors read and approved the final version of the manuscript.

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