Protein Kinase C\(\alpha\) Promotes Nicotine-induced Migration and Invasion of Cancer Cells via Phosphorylation of \(\mu\)- and m-Calpains*

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Nicotine is a major component in cigarette smoke that activates the growth-promoting pathways to facilitate the development of lung cancer. However, it is not clear whether nicotine affects cell motility to facilitate tumor metastasis. Here we discovered that nicotine potently induces phosphorylation of both \(\mu\)- and m-calpains via activation of protein kinase C\(\alpha\) (PKC\(\alpha\)), which is associated with accelerated migration and invasion of human lung cancer cells. Purified PKC\(\alpha\) directly phosphorylates \(\mu\)- and m-calpains in vitro. Overexpression of PKC\(\alpha\) results in increased phosphorylation of both \(\mu\)- and m-calpains in vivo. Nicotine also induces activation of c-Src, which is a known PKC\(\alpha\) upstream kinase. Treatment of cells with the \(\alpha\)-nicotinic acetylcholine receptor inhibitor \(\alpha\)-bungarotoxin can block nicotine-induced calpain phosphorylation with suppression of calpain activity, wound healing, cell migration, and invasion, indicating that nicotine-induced calpain phosphorylation occurs, at least in part, through a signaling pathway involving the upstream \(\alpha\)-nicotinic acetylcholine receptor. Intriguingly, depletion of PKCs by RNA interference suppresses nicotine-induced calpain phosphorylation, calpain activity, cell migration, and invasion, indicating that PKC\(\alpha\) is a necessary component in nicotine-mediated cell motility signaling. Importantly, nicotine potently induces secretion of \(\mu\)- and m-calpains from lung cancer cells into culture medium, which may have potential to cleave substrates in the extracellular matrix. These findings reveal a novel role for PKC\(\alpha\) as a nicotine-activated, physiological calpain kinase that directly phosphorylates and activates calpains, leading to enhanced migration and invasion of human lung cancer cells.

Cigarette smoking, either active or passive, is the most important risk factor in the development of lung cancer. About 90% of male and 75–80% of female lung cancer deaths in the United States are caused by smoking (1–2). Nicotine, a well-known addictive component of tobacco, acts as a tumor promoter to facilitate the outgrowth of cells with genetic damage through the nicotinic acetylcholine receptor (nAChR)\(^2\)-mediated signal transduction pathway (3–4). Lung cancers, including small cell lung cancer (SCLC) and non-small lung cancer (NSCLC), are highly metastatic tumors with poor prognosis (5). Cigarette smoking has been demonstrated to promote tumor metastasis, but the mechanism(s) remains enigmatic (6–7). The movement of cancer cells into tissue surrounding the tumor and the vasculature is the first step in the spread of metastatic cancers (8). Metastatic tumor cells have been observed to display more active motility, including migration and invasion, which appear to be a result of complex interplay between the numerous protein families participating in this process (9). Cell migration has been considered a required process during tumor cell invasion and metastasis (8, 10). Tumor invasion involves cellular migration and interaction with the microenvironment at an ectopic site (11).

Because enhanced PKC activity is frequently found in cancer cells that show highly invasive and/or metastatic potential (12), cigarette smoke constituents (i.e., nicotine) may stimulate lung tumor metastasis by regulating PKC activity. PKC is a multigene family consisting of at least 11 distinct lipid-regulated protein-serine/threonine kinases that play pivotal roles in cell growth, apoptosis, differentiation, malignant transformation, and metastasis (13). This family can be divided into three subtypes: the classic isoforms (PKC\(\alpha\), \(\beta\), \(\beta\)II, and \(\gamma\)), which are Ca\(^{2+}\) and diacylglycerol (DAG) dependent; the novel isoforms (PKC-\(\delta\), \(\varepsilon\), \(\theta\), and \(\mu\)), which are DAG dependent but Ca\(^{2+}\) independent; and the atypical isoforms (PKC-\(\xi\) and \(\lambda\)), which possess only one zinc finger and lack the characteristic C2 domain and hence are insensitive to both Ca\(^{2+}\) and DAG (14–15). PKC isoenzymes exhibit distinct tissue distribution and play a distinct role in various cellular events, including cell survival, proliferation, tumorigenesis, tumor invasion, and metastasis (6–7, 12, 16). For example, PKC\(\alpha\), an atypical PKC isoform, presents predominantly in lung and brain (17), suggesting that PKC\(\alpha\) may play a critical role in regulating lung cancer development, including metastasis.

The mammalian calpains comprise 14 family members, of which \(\mu\)-calpain (calpain 1) and m-calpain (calpain 2) are the best characterized calpain isoforms that are extensively expressed in both SCLC and NSCLC cells and are involved in the limited proteolysis of various focal adhesion structures and signaling enzymes to promote cell migration and invasion (18–19). Both \(\mu\)- and m-calpains function as heterodimeric enzymes composed of a unique, large catalytic subunit associated with a common, small regulatory subunit (calpain 4). Intriguingly, m-calpain can localize to integrin-associated adhesive structures, suggesting a potential mechanism by which m-calpain regulates cell migration (20). Studies utilizing pharmacological inhibitors and calpain knock-out cells indicate that calpain plays an important role in mediating the dynamic regulation of focal adhesions required for cell motility (21–23). In addition to Ca\(^{2+}\) binding, our recent findings and those of others reveal that phosphorylation of calpain is another essential mech-

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2 The abbreviations used are: nAChR, nicotinic acetylcholine receptor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; \(\alpha\)-BTX, \(\alpha\)-bungarotoxin; ECM, extracellular matrix; t-Boc-LM-CMCA, t-butoxy carboxy-Leu-Met-chloromethyl-aminocoumarin; PKC\(\alpha\), protein kinase C\(\alpha\); PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyloxy)pyrazolo[3,4-d]pyrimidine; siRNA, small interfering RNA; RNAi, RNA interference; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; HM, heavy membrane.
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anism for its activation (18, 24–26). Here we discovered that nicotine, a major component in cigarette smoke, can induce phosphorylation and activation of both $\mu$- and m-calpains through a novel signaling pathway involving $\alpha$-nAChR/c-Src/PKC\textsubscript{i} that contributes to accelerated wound healing, migration, and invasion of human lung cancer cells.

**EXPERIMENTAL PROCEDURES**

Materials—Anti-$\mu$-calpain, m-calpain, PKC\textsubscript{i}, c-Src, fluorescein isothiocyanate-conjugated anti-goat, and rhodamine-conjugated anti-rabbit IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nicotine and enolase were purchased from Sigma. Synthetic calpain substrate $t$-butoxy carbonyl-Leu-Met-chloromethylaminocoumarin ($t$-Boc-LM-CMAC) was obtained from Molecular Probes (Eugene, OR). The QCM\textsuperscript{TM} chemotaxis 24-well colorimetric cell migration assay kit and cell invasion assay kit were purchased from Chemicon International, Inc. (Temecula, CA). Purified PKC\textsubscript{i} was obtained from Pan-Vera (Madison, WI). PP2, $\alpha$-bungarotoxin ($\alpha$-BTX), and c-Src enzyme were purchased from Calbiochem. The PKC\textsubscript{i}/pAXneoRX construct was a kind gift from Dr. Alan P. Fields (27). All reagents used were purchased from commercial sources unless otherwise stated.

Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis—Cells were cultured with 0.5% fetal bovine serum (FBS) medium overnight. Cells were washed three times with phosphate-free RPMI 1640 medium and metabolically labeled with $[^{32}\text{P}]$orthophosphoric acid for 90 min. After treatment with nicotine or inhibitor, cells were washed with ice-cold 1× phosphate-buffered saline and lysed in detergent buffer. $\mu$- or m-calpain was immunoprecipitated using a $\mu$- or m-calpain antibody, respectively. The samples were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at $-80^\circ$ C for the time indicated. Phosphorylation of $\mu$- or m-calpain was determined by autoradiography. The same filter was then probed by Western blot analysis using a $\mu$- or m-calpain antibody, respectively, and developed by using an ECL kit from Amersham Bio-Sciences as described previously (18).

Subcellular Fractionation—Subcellular fractionation was performed to isolate heavy membrane (HM), light membrane, cytosol, and nuclear membrane as previously described (28). Protein from each fraction was subjected to SDS-PAGE and analyzed by Western blotting using $\mu$-calpain, m-calpain, and PKC\textsubscript{i} antibodies. The purity of fractions was confirmed by assessing localization of fraction-specific proteins, including prohibitin (HM; Ref. 29), caspase 3 (cytosolic; Ref. 30), and proliferating cell nuclear antigen (PCNA; Ref. 31).

PKC\textsubscript{i} Phosphorylates $\mu$- or m-Calpain in Vitro—$\mu$- or m-calpain was immunoprecipitated from lysates of H1299 cells using an agarose-conjugated $\mu$- or m-calpain antibody, respectively. The immunoprecipitated $\mu$- or m-calpain was resuspended in a kinase assay buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.1 mM CaCl\textsubscript{2}, 10 $\mu$M ATP, 40 $\mu$g/ml phosphatidylserine, and 10 $\mu$Ci of [ $^{32}$P]ATP. Purified, activated PKC\textsubscript{i} enzyme (0.1 $\mu$g) was added and incubated for 30 min at $30^\circ$ C. The reaction was stopped by the addition of 2× SDS sample buffer and boiling the sample for 5 min. The samples were analyzed by SDS-PAGE, and phosphorylation of $\mu$- or m-calpain was determined by autoradiography.

Measurement of Intracellular c-Src Activity—Cells were stimulated with nicotine or inhibitor, cells were washed with ice-cold 1× phosphate-buffered saline and lysed in detergent buffer. $\mu$- or m-calpain was immunoprecipitated using a $\mu$- or m-calpain antibody, respectively. The samples were subjected to 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at $-80^\circ$ C for 24 h. The activity of c-Src was determined by autoradiography. The same filter was then probed by Western blot analysis using a c-Src antibody.

Purified c-Src Phosphorylates PKC\textsubscript{i} in Vitro—PKC\textsubscript{i} was immunoprecipitated from cell lysates of H1299 cells and incubated with purified c-Src enzyme (Calbiochem) in the c-Src kinase assay buffer containing 2 $\mu$Ci of [ $^{32}$P]ATP at $30^\circ$ C for 10 min as described above. Phosphorylation of PKC\textsubscript{i} was determined by autoradiography.

Measurement of Intracellular PKC\textsubscript{i} Activity—Cells were treated with nicotine for various times as indicated. PKC\textsubscript{i} was immunoprecipitated from cell lysates with an agarose-conjugated PKC\textsubscript{i} antibody. Immunoprecipitated PKC\textsubscript{i} was washed and resuspended in 50 $\mu$l kinase assay buffer containing 50 mM Tris, pH 7.5, 10 mM MgCl\textsubscript{2}, 0.5 mM EGTA, 0.1 mM CaCl\textsubscript{2}, 10 $\mu$M ATP, 40 $\mu$g/ml phosphatidylserine, 10 $\mu$g of his-tone-1, and 10 $\mu$Ci of [ $^{32}$P]ATP. The reactions were incubated at room temperature for 30 min and terminated by addition of SDS sample buffer and boiling prior to SDS-polyacrylamide gel electrophoresis. The activity of PKC\textsubscript{i} was determined by autoradiography.

Vector-based Gene Silencing of PKC\textsubscript{i} by RNA Interference (RNAi)—The PKC\textsubscript{i}DNA target sequence for siRNA design is AACTTCTTGAAGAACATGCGCA. This was determined by an Ambion siRNA Target Finder according to the human PKC\textsubscript{i} cDNA sequence. The PKC\textsubscript{i}-specific hairpin siRNA insert (sense-loop-antisense) was determined using a computerized insert design tool based on a target sequence following instructions from the Ambion website. Then, the oligonucleotide encoding the PKC\textsubscript{i}-specific hairpin siRNA insert was synthesized and ligated into pSilencer\textsuperscript{TM} 2.1-U6 hygro vector from Ambion (Austin, TX). The pSilencer\textsuperscript{TM} 2.1-U6 hygro plasmids containing the PKC\textsubscript{i} hairpin siRNA were transfected into H1299 cells using Lipofectamine\textsuperscript{TM} 2000 according to the manufacturer’s instructions. The stable clones persistently producing PKC\textsubscript{i} siRNA were selected using hygromycin (0.8 mg/ml). The levels of PKC\textsubscript{i} expression were analyzed by Western blot using a PKC\textsubscript{i} antibody.

Detection of Calpain Activity in Living Cells—H1299 or H460 cells were plated at 50–80% confluence in a glass chamber and incubated with 0.5% FBS medium for 24 h. The cells were then treated with nicotine or inhibitor in the presence of F-Boc-LM-CMAC (30 $\mu$m) for 30–60 min. Samples were observed under a fluorescence microscope (excitation 329 nm, emission 409 nm) as described (24–25).

Cell Migration and Invasion Assay—Cells were treated with nicotine or inhibitor as indicated. Cell migration was assessed using a QCM\textsuperscript{TM} 24-well colorimetric cell migration assay kit (Chemicon) following the manufacturer’s instructions. This new technique does not require cell labeling, scraping, washing, or counting. Cells that migrated through the polycarbonate membrane were incubated with “Cell Stain Solution” and then subsequently extracted and detected on a standard microplate at 560 nm. Cell invasion was assessed using the Chemicon cell invasion assay kit. This assay was performed in an invasion chamber, which is a 24-well tissue plate with 12 cell culture inserts. The inserts contain an 8-$\mu$m pore size polycarbonate membrane over which a thin layer of ECMatrix\textsuperscript{TM} is dried. The extracellular matrix (ECM) layer occludes the membrane pores, blocking non-invasive cells from migrating through. Invasion cells migrate through the ECM layer and cling to the bottom of the polycarbonate membrane. The insert membrane with invaded cells on the bottom was placed in the wells with cell stain/dissociation solu-
FIGURE 1. Nicotine induces phosphorylation of μ- and m-calpains in association with increased calpain activity and accelerated wound healing, migration, and invasion of human lung cancer cells. A, expression levels of μ-calpain, m-calpain, PKCθ, and c-Src in various lung cancer cell lines were analyzed by Western blot. B, H1299 cells expressing high levels of endogenous μ- and m-calpains were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine (0.2 μM) for various times as indicated. μ- or m-Calpain was immunoprecipitated using μ- or m-calpain antibody, respectively. Phosphorylation of μ- or m-calpain was determined by autoradiography (upper). Western blot analysis using μ- or m-calpain antibody was performed to confirm and quantify μ- or m-calpain protein (lower). C, H1299 cells were incubated with 0.5% FBS medium for 24 h. The cells were then treated with nicotine (0.2 μM) in the presence of t-butoxy carbonyl-Leu-Met-chloromethyl ketone (30 μM) for 60 min. Calpain activity was analyzed by fluorescent microscopy. D, H1299 cells were seeded into a six-well tissue culture dish and allowed to grow to 90% confluence in complete medium. Cell monolayers were wounded by a plastic tip (1 mm) that touched the plate. Wounded monolayers were then washed four times with medium to remove cell debris and incubated in 0.5% FBS medium in the absence or presence of nicotine (0.2 μM) for various times up to 24 h. Cells were monitored under a microscope equipped with a camera (Deiss). E, H1299 cells were treated with nicotine (0.2 μM) for 24 h. Cell migration or invasion was measured using a QCM™ 24-well colorimetric cell migration assay kit or cell invasion kit, respectively. Each experiment was repeated three times, and data represent the mean ± S.D. of three determinations.

RESULTS

Nicotine Induces Phosphorylation and Activation of μ- and m-Calpains in Association with Accelerated Wound Healing, Migration, and Invasion of Human Lung Cancer Cells—μ- and m-Calpains are two major calpain family members that are widely expressed in both SCLC and NSCLC cells (Fig. 1A). Recently, reports indicate that epidermal growth factor-induced calpain phosphorylation enhances calpain activity in association with increased cell migration (24, 34). To test whether nicotine can mimic a growth factor to regulate calpain, human lung cancer H1299 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine (0.2 μM) for various times. Phosphorylation of μ- and m-calpains was analyzed as described under "Experimental Procedures." Results reveal that nicotine potently stimulates phosphorylation of both μ- and m-calpains in human lung cancer cells within 60–120 min (Fig. 1B). To test whether nicotine-induced calpain phosphorylation enhances its proteolytic activity, calpain activity in living cells was performed using t-butoxy carbonyl-Leu-Met-chloro-
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methyl-aminocoumarin (t-Boc-LM-CMAC, a synthetic calpain substrate) as described (18, 25). Cleavage of t-Boc-LM-CMAC by active calpains can induce retention of the chloromethylaminocoumarin portion of the molecule in the cells and can result in increased fluorescence (25). H1299 cells were treated with nicotine (0.2 \(\mu\)M) in the presence of t-Boc-LM-CMAC for 60 min. Calpain activity was assessed by fluorescence microscopy. Results indicate that exposure of cells to nicotine enhances calpain activity (Fig. 1C). Recent reports have demonstrated that phosphorylation of calpain is able to increase its activity (18, 25).

Thus, nicotine-induced calpain activation may occur by a mechanism involving its phosphorylation. Other human lung cancer cell lines (i.e. H69, H82, H157, and H460 cells) that express various levels of endogenous \(\mu\)- and m-calpains were also tested, and similar results were obtained (data not shown).

Because calpains are involved in regulating cell migration and invasion (21, 26), nicotine-induced phosphorylation and activation of calpains may promote migration and invasion of human lung cancer cells. To test this possibility, a wound-healing assay was employed as described under "Experimental Procedures." Compared with control cells, wound repair is significantly accelerated following treatment with nicotine (Fig. 1D). Cell migration or invasion analysis using a QCMTM chemotaxis 24-well colorimetric cell migration or invasion assay kit revealed that nicotine significantly enhances both migration and invasion of human lung cancer cells (Fig. 1E).

Collectively, these findings suggest that nicotine-stimulated migration and invasion of lung cancer cells may occur, at least in part, through phosphorylation and activation of calpains.

PKC\(\alpha\) Co-localizes and Interacts with \(\mu\)- and m-Calpains in Cytoplasm—PKC\(\alpha\) is extensively co-expressed with \(\mu\)- and m-calpains in both SCLC and NSCLC cells (Fig. 1A). To assess a potential direct role for PKC\(\alpha\) as a physiological calpain kinase, subcellular distribution of PKC\(\alpha\) and calpains was examined by subcellular fractionation assay.

Results demonstrate that PKC\(\alpha\) is co-localized with \(\mu\)- or m-calpain in heavy membrane, light membrane, and cytosol in human lung cancer H1299 cells (Fig. 2A). To verify the purity of the subcellular fractions obtained, fraction-specific proteins were assessed by probing the same filters. Prohibitin, an exclusively mitochondrial protein (29), was detected only in the HM fraction. CPP32 (caspase 3), which is a cytosolic protease in growing cells (30), was detected exclusively in the cytosol, whereas proliferating cell nuclear antigen, which is a nuclear marker (31), was detected exclusively in the nuclear fraction (Fig. 2A). These data reveal that each fraction obtained is highly pure without cross-contaminiation. To test whether nicotine stimulates an association between PKC\(\alpha\) and calpain, H1299 cells were treated with nicotine (0.2 \(\mu\)M) for various times. A co-immunoprecipitation experiment was carried out using an agarose-conjugated PKC\(\alpha\) antibody, respectively. Results indicate that nicotine induces activation of PKC\(\alpha\) with a peak between 60–120 min (Fig. 3). Intriguingly, the peak time of PKC\(\alpha\) activity is consistent with the kinetics of nicotine-stimulated calpain phosphorylation (60–120 min; Fig. 1B), suggesting a role of PKC\(\alpha\) in nicotine-induced calpain phosphorylation. To test whether activated PKC\(\alpha\) can directly phosphorylate \(\mu\)- and m-calpains, \(\mu\)- or m-calpain protein was immunoprecipitated from H1299 cells and incubated with purified, active PKC\(\alpha\) in a kinase assay buffer containing \([^{32}P]ATP\) as described under "Experimental Procedures." Importantly, active PKC\(\alpha\) directly phosphorylates both \(\mu\)- and m-calpains in vitro (Fig. 3B). To determine whether PKC\(\alpha\) is a calpain kinase in vivo, a PKC\(\alpha\)\(^{\Delta}\)XneoRX expression construct or vector-only control was transfected into H460 cells that express relatively low levels of endogenous PKC\(\alpha\). After transfection for 48 h, cells were metabolically labeled with \([^{32}P]\)orthophosphoric acid and treated with nicotine (0.2 \(\mu\)M) for 60 min. Results indicate that overexpression of PKC\(\alpha\) not only enhances nicotine-induced phosphorylation of \(\mu\)- and m-calpains but also increases nicotine-stimulated migration and invasion of human lung cancer cells (Fig. 4). These findings provide both biochemical and genetic evidence that \(\mu\)- and m-calpains are novel physiological PKC\(\alpha\) substrates in nicotine-activated cell migration and invasion signaling.

Nicotine Stimulates Activation of c-Src, Which Can Directly Phosphorylate and Activate PKC\(\alpha\), and the Specific Src Inhibitor PP2 Suppresses Nicotine-stimulated Phosphorylation and Activation of \(\mu\)- and m-Calpains in Association with Decreased Cell Migration and Invasion—Our data show that nicotine induces activation of PKC\(\alpha\), which can directly phosphorylate calpains (Figs. 3 and 4). However, the upstream protein kinase(s) for PKC\(\alpha\) activation remains unclear. PKC\(\alpha\) is insensitive to

![Figure 2. PKC\(\alpha\) co-localizes and interacts with \(\mu\)- and m-calpains in cytoplasm. A. subcellular fractionation as previously described (28) was performed to isolate heavy membrane (HM), light membrane (LM), cytosol (Cyt), and nuclear membrane (Nuc) from H1299 cells. Western blot analysis of subcellular fractions was performed to detect \(\mu\)-calpain, m-calpain, and PKC\(\alpha\). The purity of fractions was confirmed by assessing localization of fraction-specific proteins, including prohibitin (HM; Ref. 29), caspase 3 (cytosolic; Ref. 30), and proliferating cell nuclear antigen (PCNA; Ref. 31). B. H1299 cells were treated with nicotine (0.2 \(\mu\)M) for various times as indicated. A co-immunoprecipitation experiment was performed using an agarose-conjugated \(\mu\)- or m-calpain antibody, respectively. The \(\mu\)- or m-calpain-associated PKC\(\alpha\) and total \(\mu\)- or m-calpain were analyzed by Western blot.](image-url)
Ca\(^{2+}\) due to the absence of the calcium-binding domain (16). Thus, nicotine-induced activation of PKC\(\alpha\) may occur through a calcium-independent mechanism. Because c-Src has been reported to directly induce tyrosine phosphorylation of PKC\(\alpha\) at tyrosine 256, 271, and 325 sites along with activation of enzyme activity (32), and c-Src is ubiquitously expressed in both SCLC and NSCLC cells (Fig. 1A), we postulate that nicotine may stimulate c-Src activity to phosphorylate and activate PKC\(\alpha\). To test this, H1299 cells expressing endogenous c-Src were treated with nicotine for various times, followed by immunoprecipitation of c-Src and measurement of its activity by an immune complex kinase assay with acid-treated enolase as a substrate. Results reveal that nicotine potently enhances c-Src activity in a time-dependent manner (Fig. 5A). To test whether c-Src can directly phosphorylate endogenous PKC\(\alpha\), PKC\(\alpha\) protein was immunoprecipitated from H1299 cells and incubated with purified, active c-Src in a kinase assay buffer containing \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) as described under “Experimental Procedures.” Results indicate that active c-Src directly phosphorolysed PKC\(\alpha\) in vitro (Fig. 5B). These findings suggest that c-Src may function as an upstream PKC\(\alpha\) kinase in nicotine-stimulated cell migration and invasion signaling. To pharmacologically test this, H1299 cells were treated with nicotine in the absence or presence of increasing concentrations of the Src-specific inhibitor PP2 (32). Results show that PP2 not only inhibits nicotine-induced calpain phosphorylation and activation but also potently suppresses cell migration and invasion (Fig. 6). Collectively, these findings suggest that nicotine-induced migration and invasion of human lung cancer cells may occur by a mechanism involving the c-Src/PKC\(\alpha\)/calpain signal pathway.

The \(\alpha\)-\(n\)AChR-specific Inhibitor \(\alpha\)-BTX Inhibits Nicotine-induced Phosphorylation and Activation of \(\mu\)- and \(m\)-Calpains in Association with Suppression of Migration, Invasion, and Wound Healing of Human Lung Cancer Cells—\(\alpha\)-BTX has been identified as the site-selective antagonist for \(\alpha\)-\(n\)AChR (36). Because \(\alpha\)-\(n\)AChR plays an important role in lung cancer cell signaling (36), we tested whether \(\alpha\)-BTX affects nicotine-induced phosphorylation of \(\mu\)- and \(m\)-calpains in H1299 lung cancer cells. Results indicate that \(\alpha\)-BTX potently blocks nicotine-induced phosphorylation of \(\mu\)- and \(m\)-calpains in association with decreased calpain activity, cell migration, and invasion (Fig. 7A–C). Importantly, \(\alpha\)-BTX potently inhibits nicotine-stimulated wound healing (Fig. 7D). These findings suggest that \(\alpha\)-\(n\)AChR functions as the upstream receptor in nicotine-induced calpain phosphorylation as well as migration and invasion of human lung cancer cells.

Depletion of PKC\(\alpha\) Suppresses Nicotine-induced Calpain Phosphorylation, Migration, and Invasion of Human Lung Cancer Cells—Our findings suggest that PKC\(\alpha\) functions as a nicotine-activated calpain kinase.
in human lung cancer cells (Figs. 1–4). To test whether PKC\(\alpha\) is essential for nicotine-stimulated calpain phosphorylation, a vector-based stable gene silencing approach was employed for specific depletion of PKC\(\alpha\) from human lung cancer cells. The pSilencer\textsuperscript{TM} 2.1-U6 hygro plasmids bearing the PKC\(\alpha\)-specific siRNA were transfected into H1299 cells. The stable clones persistently producing PKC\(\alpha\) siRNA were selected using hygromycin. Results indicate that cells expressing PKC\(\alpha\) siRNA display >90% reduction of PKC\(\alpha\) protein expression (Fig. 8A). Importantly, specific disruption of PKC\(\alpha\) expression by RNAi blocks nicotine-induced phosphorylation of \(\mu\)- and m-calpains in association with suppression of nicotine-stimulated calpain activity, cell migration, invasion, and wound healing (Fig. 8). These findings indicate that PKC\(\alpha\) is an essential kinase for nicotine-induced calpain phosphorylation, cell migration, and invasion.

**Nicotine Enhances Secretion of Calpains from Human Lung Cancer Cells, Which Could Be Blocked by PKC\(\alpha\)-specific siRNA.** From Fig. 1A, it is observed that nicotine potently enhances secretion of both \(\mu\)- and m-calpains within 24 h (Fig. 9). Intriguingly, the \(\alpha\)-nAChR inhibitor \(\alpha\)-BTX and the Src-specific inhibitor PP2 not only suppress nicotine-induced calpain phosphorylation (Figs. 6 and 7) but also significantly block secretion of both \(\mu\)- and m-calpains (Fig. 9). These findings suggest that nicotine-induced calpain phosphorylation may affect calpain secretion.

**DISCUSSION**

Cigarette smoke is by far the number one cause of lung cancer, and ~90% of lung cancer occurs in smokers or former smokers (5). Tumor progression to the invasive and metastatic states dramatically enhances the mortality of cancer. Lung cancer has a very poor prognosis because of its high metastatic potential. The liver and adrenal gland have been found to be frequent sites of spread from lung cancer (5). Rational therapeutic interventions will only be possible when we understand the molecular mechanisms governing cellular behavior underlying this transformation. For invasion, a subpopulation of tumor cells must recognize, modify, and migrate through the ECM barrier and then proliferate in the adjacent but ectopic locale (39). Therefore, the proteolytic ability of the cell is a key factor in the processes of cell migration and invasion. Calpain has been reported to be a positive regulator of cell migration and invasion because it localizes to focal adhesions and cleaves many focal adhesion-related proteins in the ECM, including integrin receptors, focal adhesion kinase, and talin, etc. (40). Intriguingly, calpain activity is significantly elevated in various tumor cells when compared with nonmalignant cells (41–42). A recent in vivo study indicates that antisense-mediated suppression of m-calpain inhibited invasion of prostate carcinoma cells (11). Thus, elevated calpain activity may enhance motility of tumor cells, which may potentially promote tumor metastasis.

Growing evidence indicates that cigarette smoking facilitates the spread of cancer in the body in various types of cancer, including human lung and breast cancers (6–7). However, the intracellular signal mechanism by which cigarette smoking promotes metastasis and/or development of lung cancer remains elusive. Here we discovered that nicotine, a major component in cigarette smoke, can stimulate phosphorylation of \(\mu\)- and m-calpains with increased proteolytic activity in human lung cancer cells (Fig. 1). Functionally, treatment of lung cancer cells with nicotine (0.2 \(\mu\)M) results in accelerated wound healing and enhanced cell migration and invasion (Fig. 1, D and E). Phosphorylation of calpain has been demonstrated to positively regulate its proteolytic activity (18, 25–26). Thus, nicotine-stimulated cell motility signaling may occur in a mechanism through phosphorylation of \(\mu\)- and m-calpains that enhances their activities. Because nicotine exists at high concentrations (0.09–1 \(\mu\)M) in the blood of smoking patients (44), the concentration of nicotine (0.2 \(\mu\)M) in our experiments falls within the range found in the blood of smoking patients.

PKC\(\alpha\) is an atypical PKC isoform, and Northern blot analysis, using the full-length PKC\(\alpha\) cDNA as a probe, revealed that the PKC\(\alpha\) transcript presents predominantly in lung and brain (17). Consistently, our data show that PKC\(\alpha\) is ubiquitously expressed in both human SCLC and NSCLC cells (Fig. 1A). It is possible that PKC\(\alpha\) may play a pivotal role in regulating lung cancer metastasis. It is known that PKC is implicated in tumor invasion and metastasis (12), but the downstream substrate for this effect is not clear. Evidence reported here suggests that PKC\(\alpha\) functions as a calpain kinase because PKC\(\alpha\) co-localizes and interacts with \(\mu\)- and m-calpains in the cytoplasm and directly phosphorylates either \(\mu\)- or m-calpain in vitro, indicating its potential, direct role as a calpain kinase (Figs. 2 and 3). Confirmation of PKC\(\alpha\) as a physiological calpain...
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kinase was obtained in vivo from results of transfection studies demonstrating that PKC\(\alpha\), when overexpressed in H460 cells, resulted in enhanced phosphorylation of \(\mu\)- and m-calpains, which are consistent with in vitro results (Fig. 4). Importantly, specific knockdown of PKC\(\alpha\) expression by RNAi can significantly inhibit nicotine-stimulated calpain phosphorylation and activity in association with decreased migration and invasion of H1299 lung cancer cells (Fig. 8). These findings strongly indicate that PKC\(\alpha\) functions as a physiological nicotin-stimulated calpain kinase that positively regulates calpain activity through phosphorylation, leading to migration and invasion of human lung cancer cells.

PKC\(\alpha\) belongs to an atypical PKC isoenzyme category that differs significantly from other PKC family members in their regulatory domain in that it lacks both the calcium-binding domain and one of the two zinc finger motifs required for DAG binding (16). These domain variations result in a different requirement for activation. Because PKC\(\alpha\) is insensitive to both Ca\(^{2+}\) and DAG (16), other mechanisms, for example, phosphorylation or protein-protein interaction, may be required for PKC\(\alpha\) activation. Recent studies reveal that c-Src not only induces tyrosine phosphorylation of PKC\(\alpha\) but also directly binds to PKC\(\alpha\), which leads to its activation (32). Because nicotine can induce activation of c-Src, which could then in turn phosphorylate PKC\(\alpha\) (Fig. 5), these findings strongly suggest that c-Src most likely functions as a nicotine-activated upstream PKC\(\alpha\) kinase.

**FIGURE 7.** The \(\alpha_2\) nAChR-specific inhibitor \(\alpha\)-bungarotoxin (\(\alpha\)-BTX) inhibits nicotine-induced phosphorylation and activation of \(\mu\)- and m-calpains in association with suppression of migration, invasion, and wound healing of human lung cancer cells. A, H1299 cells were metabolically labeled with \([\text{\textsuperscript{32}}P]\text{orthophosphoric acid and treated with nicotine (0.2 \(\mu\)M) in the absence or presence of various concentrations of \(\alpha\)-BTX for 60 min. Phosphorylation of \(\mu\)- or m-calpain was analyzed as described in Fig. 1. B, H1299 cells were incubated with \(t\)-Boc-LM-CMAC (20 \(\mu\)M) and treated with nicotine (0.2 \(\mu\)M) in the absence or presence of \(\alpha\)-BTX (20 \(\mu\)M) for 60 min. Calpain activity was analyzed by fluorescent microscopy. C, H1299 cells were treated with nicotine (0.2 \(\mu\)M) in the absence or presence of increasing concentrations of \(\alpha\)-BTX for 24 h. Cell migration or invasion was measured using a QCM\textsuperscript{TM} 24-well colorimetric migration assay kit or cell invasion kit, respectively. Each experiment was repeated three times, and data represent the mean \pm S.D. of three determinations. D, H1299 cells were treated with nicotine (0.2 \(\mu\)M) in the absence or presence of \(\alpha\)-BTX (20 \(\mu\)M) for 24 h. Monolayer wound healing assay was performed as described in Fig. 10.
nAChRs are cationic channels whose opening is controlled by acetylcholine and nicotinic receptor agonists. The $\alpha_\text{C}$ nAChR is expressed in normal human small airway epithelial cells, SCLC and NSLC cells (36–37, 45). $\alpha$-BTX has been identified as the site-selective antagonist for $\alpha_\text{C}$, but not $\alpha_\beta$, nAChRs (36, 46, 47). Because $\alpha$-BTX potently blocks both nicotine-induced phosphorylation and activation of $\mu$- and m-calpains.
in association with inhibition of wound healing, cell migration, and invasion (Fig. 7), this indicates that nicotine-induced cell migration and invasion may occur through activation of the α7 nAChR signal transduction pathway involving α7 nAChR/c-Src/PKCα/βII/PKC♂ calpains in lung cancer cells. Importantly, α7-BTX may have potential clinical relevance in strategies designed to restrain tumor invasion and metastasis through this novel mechanism in patients with lung cancer.

In addition to PKC♂, our previous report and others have demonstrated that mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 can function as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) sensors (26). Therefore, nicotine-induced calpain phosphorylation through PKC♂ activates PKC♂ kinases may cooperatively regulate calpain activity through its phosphorylation. However, PKC♂ may play a more extensive and/or more important role in nicotine-stimulated cell migration and invasion signaling because depletion of PKC♂ by RNAi not only blocks nicotine-induced calpain phosphorylation but also significantly suppresses migration and invasion of human lung cancer cells (Fig. 8).

Calpain was generally believed to exist and function only in the cytoplasm. Recent studies indicate that m-calpain has also been detected in the extracellular space of tissue that results from active secretion rather than cell destruction (43). We have previously demonstrated that treatment of SCLC H69 cells with nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)- or epidermal growth factor-activated kinases 1 and 2 can function as nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-responsive kinases. Thus, various types of calpain kinases and secretion of C-BTX or PP2, and data represent the mean ± S.D. of three determinations.

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