Protein Kinase C Controls Vesicular Transport and Secretion of Apolipoprotein E from Primary Human Macrophages*

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Background: Macrophage-specific apolipoprotein E (apoE) secretion has been implicated as protective in atherosclerosis. The precise signaling mechanisms regulating apoE secretion from primary human monocyte-derived macrophages (HMDMs) remain unclear. Here we investigate the role of protein kinase C (PKC) in regulating basal and stimulated apoE secretion from HMDMs. Treatment of HMDMs with structurally distinct pan-PKC inhibitors (calphostin C, Ro-31-8220, Go6976) and a PKC inhibitory peptide all significantly decreased apoE secretion without significantly affecting apoE mRNA or apoE protein levels. The PKC activator phorbol 12-myristate 13-acetate (PMA) stimulated apoE secretion, and both PMA-induced and apoAI-induced apoE secretion were inhibited by PKC inhibitors. PKC regulation of apoE secretion was found to be independent of the ATP binding cassette transporter ABCA1. Live cell imaging demonstrated that PKC inhibitors inhibited vesicular transport of apoE to the plasma membrane. Pharmacological or peptide inhibitor and knockdown studies indicate that classical isoforms PKCa/β and not PKCδ, -ε, -θ, or -ζ isoforms regulate apoE secretion from HMDMs. The activity of myristoylated alanine-rich protein kinase C substrate (MARCKS) correlated with modulation of PKC activity in these cells, and direct peptide inhibition of MARCKS inhibited apoE secretion, implicating MARCKS as a downstream effector of PKC in apoE secretion. Comparison with other secreted proteins indicated that PKC similarly regulated secretion of matrix metalloproteinase 9 and chitinase-3-like-1 protein but differentially affected the secretion of other proteins. In conclusion, PKC regulates the secretion of apoE from primary human macrophages.

Apolipoprotein E (apoE) is a 34-kDa multifunctional protein that plays a critical role in processes relevant to atherosclerosis (1–3). ApoE mediates the clearance of triglyceride-rich lipoproteins, inhibits proliferation of smooth muscle cells and lymphocytes, contributes to antigen presentation, and promotes cholesterol efflux from foam cell macrophages (3–9). ApoE knock-out mice develop hyperlipidemia and severe atherosclerotic lesions (10, 11). Transplantation of apoE-expressing bone marrow into apoE knock-out mice results in a marked decrease in diet-induced atherosclerosis in these mice (12). In contrast, more recent studies demonstrate a strong association between elevated plasma apoE and increased risk of cardiovascular death in the elderly (13). These apparently disparate effects may relate to the differences in function between macrophage-derived apoE and apoE derived from other cellular sources (14) and highlight the importance of understanding the regulation of macrophage apoE secretion.

It is known that newly synthesized apoE follows the classical secretory pathway, whereby it is trafficked into the endoplasmic reticulum, post-translationally glycosylated and sialylated in the Golgi network, and transported in vesicles to the plasma membrane for secretion (1, 8, 15, 16) or reinternalization and resecretion or degradation (2, 17–19). ApoE secretion is a constitutive process controlled by the ATP-binding cassette transporter, ABCA1 (20, 21). In macrophages, apoE is synthesized in excess of cellular requirements (22). The balance between secretion and degradation of apoE depends on a range of factors, including the presence of secretory stimuli, such as high density lipoprotein (HDL) and apoAI (23–26).

Recently, our laboratory has demonstrated roles for phospholipase C, protein kinase A (PKA), intracellular calcium...
(Ca\(^{2+}\)), and protein phosphatase 2B (PP2B)\(^2\) in regulating apoE secretion from macrophages (16, 27). In macrophages, apoE-containing vesicles are associated with the microtubular network, and perturbing this network inhibits apoE secretion (16). Stimulation of PKA activity did not enhance apoE secretion, suggesting that additional pathways were active. Because PKA and protein kinase C (PKC) interact in various cell signaling networks (28, 29) and PKC is known to be activated downstream of phospholipase C (30), it is likely that PKC plays a role in apoE secretion. A previous study indirectly implicated PKC in apoE secretion by demonstrating that phorbol ester inhibited apoE secretion in mouse macrophages secondary to decreased apoE synthesis (31). However, the PKC-independent effects of prolonged phorbol 12-myristate 13-acetate (PMA) exposure complicate interpretation of these data.

A direct role for PKC in protein secretion per se is supported by its established roles in mediating the secretion of various cargoes, such as glutamate and noradrenaline from neuronal cell lines (32–35), mucin from colonic tumor cell lines (36), histamine from rat basophilic leukemia mast cells (37), and insulin and glucagon from pancreatic cells (38–41). Furthermore, PKC has been reported to interact with a number of proteins associated with intracellular transport (e.g. actin, tubulin, \(B\prime\)-COP, p62-ZIP, and myristoylated alanine-rich protein kinase C substrate (MARCKS)) (42).

PKC is a member of the serine/threonine family of kinases with at least 11 isoforms classified into three groups: classical (\(\alpha, \beta, \gamma\)), novel (\(\delta, \theta, \eta\)), and atypical (\(\zeta, \iota, \mu, \lambda\)) (30, 43). Macrophages express the \(\alpha, \beta, \delta, \epsilon, \theta, \zeta, \iota, \mu, \lambda\) and PKC isoforms (44). Clarifying the role of specific PKC isoform(s) in apoE secretion may be of particular clinical relevance because PKC activation has been observed in various diseases, and inhibition of PKC has been investigated for treatment of diabetic peripheral retinopathy (e.g. ruboxistaurin/LY333531), cancer (e.g. UCN-01, CGP41251), and psoriasis (e.g. AE0771) (45–48). The biological consequences of inhibition of PKC may be both diverse and clinically important. Given differences in the isoform expression of PKC in different cell types, data specific to primary human macrophages are important.

The present study has investigated the role of PKC in regulating the secretion of apoE from primary human macrophages. We identify for the first time likely roles for the classical PKC isoforms in this process, establish that PKC acts independently of ABCA1, and report a likely role for MARCKS as a down-stream mediator of this process.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calphostin C (CalpC), Ro-31-8220, bisindoylalmaide I (BisI), G69976, PMA, 4-\(\alpha\)-phorbol, and PKC isoform-specific inhibitory peptides (to PKCe, \(-\alpha, -\theta, \) and \(-\iota/\zeta\)) were purchased from Merck Australia. The broad PKC inhibitory peptide (fragment 19–36), BAPTA-AM, 2-aminothoxydiphenylborate (2-APB), PD98059, and SB203580 were from Sigma. BIO-11000 was synthesized by GL Biochem (Shanghai). The LY379196 compound was provided by Lilly (Grant ExNCR: B7A-AYY003). Antibodies raised against PKCa/\(\beta\), PKCb, PKCc, fibronectin, and HSP90 were from BD Biosciences. Phospho-MARCks (Ser-152/156), MARCKS, phospho-ERK44/42 (Thr-202/Tyr-204), ERK44/42, phospho-p38 MAPK (Thr-180/Tyr-182), and p38 MAPK antibodies were from Cell Signal Technology. Stealth siRNA, non-silencing control, and RNAiMax were from Invitrogen. Human apoAI, acetylated LDL, and lipoprotein-deficient serum were all prepared as described previously (49). The apoE-green fluorescent protein (GFP) construct was generated as described previously (16).

**Culture of Human Monocyte-derived Macrophages (HMDMs) and Inhibitor Treatment**—Human monocytes were isolated through density gradient centrifugation from buffy coat preparations from healthy donors of the New South Wales Red Cross and differentiated for 7–9 days into HMDMs as described previously (26). For inhibitor treatment and pulse-chase experiments, HMDMs were enriched with cholesterol by incubating them with RPMI 1640 medium supplemented with 10% (v/v) lipoprotein-deficient serum and 50 \(\mu\)g/ml acetylated LDL for 2 days to maximize apoE synthesis (26, 50–54). For inhibitor experiments, HMDMs were incubated with the indicated concentrations of PKC inhibitors or corresponding vehicle (DMSO) control in RPMI medium containing 0.1% (w/v) BSA. Levels of secreted apoE in the medium were measured by ELISA and/or Western blot, and cellular apoE protein levels were analyzed by Western blot, as described previously (16). Western blots were quantified using ImageJ version 1.42b (21). Total RNA from HMDMs was isolated using TRISure and quantified by real-time RT-PCR as described previously (21). All experiments were conducted in triplicate cultures, and treatments were expressed relative to control. All experiments were repeated using multiple independent donors of primary HMDMs.

**Live Cell Imaging and Analysis**—Cultured HMDMs (2 \(\times 10^5\) cells/ml) were transiently transfected with 3–5 \(\mu\)g of apoE-GFP cDNA using the Amaxa transfection system according to the manufacturer’s instructions and incubated overnight. HMDMs were treated with 200 nM CalpC or 5 \(\mu\)M Ro-31-8220 for 10–20 min prior to imaging. Live cell imaging of apoE-GFP-positive cells for 3–5 min was performed using a Leica Microsystems TCS SP5 confocal laser-scanning microscope equipped with a \(\times 63\) water immersion lens and a heated stage. The vesicular speed of 100 representative apoE-GFP-containing vesicles from at least two independent buffy coat preparations of HMDMs was quantified using Imaris software (Bitplane AG).

**siRNA Knockdown Experiments**—HMDMs were transfected with Stealth siRNA (Invitrogen) targeting PKCa/\(\beta\) or PKCc isoforms using RNAiMax transfection reagent (Invitrogen) according to the manufacturer’s instructions and incubated for 2 days. Cells were cultured in RPMI + 10% (v/v) human serum for a further 2–4 days and then washed and incubated with RPMI medium containing 0.1% (w/v) BSA for 1 h prior to harvesting medium and cells. Levels of PKC isoform expression were determined by Western blot analysis and real-time PCR.
Cellular apoE and secreted apoE were detected using Western blot analysis.

Cell Viability—Cell viability was routinely assessed by measuring the leakage of lactate dehydrogenase into the medium as previously described (21) and was greater than 80% for all experiments.

PKC Activity Assays—In preliminary experiments, PKC activity was determined using the PKC assay kit (Millipore) as per the manufacturer’s instructions in order to confirm that concentrations of inhibitors were effective in HMDMs. The PKC inhibitors BisI, CalpC, Ro-31-8220, and Gö6976 inhibited PKC activity by between 28 and 95%, depending on the specific inhibitor and concentration, whereas acute exposure to PMA (40 min at 250 nM) increased PKC activity (by a mean of 295%) (data not shown).

Statistical Analysis—Data are presented as the mean ± S.E. of the indicated number of independent experiments or the mean ± S.D. of one experiment representative of at least two separate experiments, each experiment using independent HMDM donors. Data were analyzed by non-parametric or parametric tests as appropriate. For non-parametric data, significant difference between control and multiple treatment groups was assessed by the Kruskal-Wallis test, and comparison of two groups was performed by the Mann-Whitney U test. Time course studies were analyzed using two-way analysis of variance with treatment as the between-group effect and time as the within-group effect. Analyses were performed using GraphPad Prism version 6.0. A two-tailed p < 0.05 was considered statistically significant.

RESULTS

Structurally Distinct Pan-PKC Inhibitors Inhibit Basal ApoE Secretion from HMDMs—To investigate whether PKC plays a role in apoE secretion from HMDMs, the effect of several pan-PKC inhibitors with differing sites of action was investigated. CalpC is a light-sensitive perylene quinone PKC inhibitor that targets the PKC regulatory domain (55, 56). Both Ro-31-8220 and BisI target the PKC catalytic domain containing the ATP binding site (45, 55). The PKC peptide fragment 19–36 inhibits PKC by binding to its pseudosubstrate domain. BisI (data not shown), CalpC (Fig. 1A), and Ro-31-8220 (Fig. 1B) all caused a rapid (within 1 h) dose-dependent decrease in apoE secretion from cholesterol-loaded HMDMs. The PKC inhibitory peptide also significantly inhibited apoE secretion, albeit less effectively (Fig. 1C). The effect of CalpC and Ro-31-8220 was similar in HMDMs that were not cholesterol-enriched (supplemental Fig. 1). Although CalpC, Ro-31-8220 and PKC inhibitory peptide significantly inhibited apoE secretion, they only minimally affected cellular levels of apoE protein (supplemental Fig. 2 A–C, respectively) and mRNA (supplemental Fig. 2, D–F, respectively), suggesting a direct effect on secretion rather than an effect secondary to decreased synthesis.

To determine whether PKC inhibition exerted a direct effect on apoE secretion independent of ongoing apoE synthesis, pulse-chase experiments were performed as described previously (16, 21). In brief, cholesterol-loaded HMDMs were incubated with [35S]methionine for 3 h in methionine/cysteine-free medium, followed by a chase in methionine/cysteine-replete medium not containing [35S]methionine, and levels of cell-associated and secreted [35S]apoE were determined and expressed relative to starting material (Fig. 2). Despite donor-
specific differences in the rates of apoE secretion (compare controls in Fig. 2, A and D), both CalpC and Ro-31-8220 consistently and directly inhibited secretion of [35S]apoE within 1 h by 58% (from 20.7 ± 1.2 to 8.7 ± 2.5% secretion; Fig. 2A; p < 0.001) and 51% (from 10.6 ± 0.4 to 5.1 ± 0.5% secretion; Fig. 2D; p < 0.001), respectively. There were no detectable effects on levels of cell-associated apoE (Fig. 2, B and E) or calculated degradation during PKC inhibition (Fig. 2, C and F).

PKC Inhibition Immobilizes ApoE-containing Vesicles in Human Macrophages—ApoE is extensively glycosylated, and the presence of multiple glycoforms on two-dimensional electrophoresis is an indicator of normal transport and processing through the Golgi and trans-Golgi network (15, 57). Two-dimensional electrophoresis analysis of cellular apoE after PKC inhibition showed no significant change in the glycoform profile (supplemental Fig. 3), suggesting that PKC inhibition effects are post-Golgi.

We have previously reported that in murine RAW macrophages, apoE is transported to the plasma membrane in discrete vesicles and that inhibition of PKA leads to immobilization of the movement of these vesicles (16), whereas PP2B inhibition had no effect (16, 27). To investigate the effect of PKC inhibition on such vesicular transport, human macrophages were transiently transfected with apoE-GFP cDNA. Live cell imaging of control HMDMs demonstrated rapid, multidirectional movement of apoE-containing vesicles, including movement to the plasma membrane (supplemental Movies 1 and 3 and Fig. 3A). This movement was arrested in the presence of 200 nM CalpC (supplemental Movie 2 and Fig. 3B) and 5 μM Ro-31-8220 (supplemental Movie 4). Quantification of the speed of vesicular movement in control versus CalpC- or Ro-31-8220-treated cells demonstrated a marked decrease in the average speed of apoE-containing vesicles from 0.42 μm/s in control cells to 0.14 μm/s (CalpC; p < 0.0001) and 0.15 μm/s (Ro-31-8220; p < 0.0001) in cells treated with PKC inhibitors (Fig. 3C). This result supports a direct effect of PKC inhibition on apoE vesicular traffic and, in this respect, indicates that PKC is similar to PKA and dissimilar to PP2B in its site of regulation of apoE secretion in macrophages.

Stimulation of ApoE Secretion by ApoAI Is PKC-dependent and ABCA1-independent—ApoAI stimulates apoE secretion by redirecting apoE away from degradation toward secretion (21, 25). Previous studies indicated that apoAI activates phospholipase C, which in turn activates PKCa, and that HDL stimulates PKC during its stimulation of cholesterol efflux from cells (58–60). However, it is unclear whether PKC regulates apoAI-mediated apoE secretion. In order to investigate this, HMDMs were pretreated with PKC inhibitor CalpC (Fig. 4A) or Ro-31-8220 (data not shown) prior to the addition of apoAI. The stimulation of apoE secretion by apoAI was markedly inhibited by pretreatment with CalpC. CalpC decreased basal and apoAI-stimulated apoE secretion to similar extents.
FIGURE 3. Inhibition of PKC arrests vesicular movement of apoE-containing vesicles. HMDMs were transiently transfected with apoE-GFP cDNA and cultured for 24 h prior to performing microscopy in RPMI containing 0.1% BSA. Individual cells expressing apoE-GFP were identified using confocal Leica SP5 microscope on a heated stage, and GFP-positive vesicles were tracked and average speed was determined after exposure to CalpC (200 nM) or Ro-31-8220 (5 μM) for 10–20 min prior to imaging. A and B show a representative sequence of still images tracking apoE-GFP vesicles of the same cell before (A) and after (B) the addition of CalpC. Average speed (C) of vesicles was obtained from 100 representative vesicles from at least two independent macrophage preparations from different donors. Statistical analysis was performed using the Mann-Whitney U test with p < 0.0001 relative to control.
Classical PKC Isoforms Regulate ApoE Secretion

**RESULTS**

**PKC Activation by PMA Increases ApoE Secretion**—Our previous studies identified that stimulation of PKA activity failed to stimulate apoE secretion above basal levels (16). The effect of PKC stimulation was investigated using brief exposure to PMA, which activates classical and novel PKC isoforms by binding to phorbol, which does not affect PKC activity, also had no effect on apoE secretion (data not shown). To investigate whether PKC, PP2B, and Ca\(^{2+}\) were involved in PMA-stimulated apoE secretion, we preincubated HMDMs with the PKA inhibitor H89, the PP2B inhibitor cyclosporin A, the intracellular Ca\(^{2+}\) chelator BAPTA-AM, and the inositol 1,4,5-trisphosphate receptor antagonist 2-APB prior to stimulation with PMA. PMA-mediated apoE secretion was PKA-dependent (Fig. 5D), PP2B-dependent (Fig. 5E), and Ca\(^{2+}\)-dependent (Fig. 5F), suggesting that PKC acts upstream of PKA and PP2B and is independent on intracellular Ca\(^{2+}\).

Because secreted apoE is lipidated (61), it is possible that PKC activity regulates apoE secretion qualitatively by affecting the lipiddation of secreted apoE. ApoE lipiddation was measured indirectly by measuring the densities of secreted apoE using a sucrose gradient as described previously (27). Stimulation of apoE secretion using PMA or inhibition of apoE secretion using Ro-31-8220 or CalpC did not significantly affect the density of secreted apoE (1.02–1.20 g/ml) (supplemental Fig. 4). Thus, the regulation of apoE secretion by PKC is probably distinct from the processes regulating its lipiddation. In other experiments, acute inhibition of PKC using Ro-31-8220 or CalpC and its acute stimulation with PMA had no demonstrable effects on the hydrolysis of VLDL triglyceride by primary macrophages or on the release of cholesterol from cholesterol-enriched primary macrophages (data not shown).

**Classical PKC Isoforms, Most Likely PKC\(\alpha/\beta\), Are Involved in Basal ApoE Secretion**—Macrophages express the classical PKC isoforms \(\alpha\) and \(\beta\); the novel PKC isoforms \(\delta,\epsilon,\) and \(\theta\); and the atypical PKC isoforms \(\zeta,\iota,\) and \(\lambda\) (44). The pan-PKC inhibitors used in Fig. 1 (CalpC, Bisl, and Ro-31-8220) target both classical and novel PKC isoforms. To distinguish between classical and novel isoforms, we investigated the effect of the relatively specific classical isoform (PKC\(\alpha/\beta\) and -\(\gamma\)) inhibitor Gö6976 (55, 64) on basal apoE secretion. Similar to the pan-PKC inhibitors, Gö6976 induced a dose-dependent decrease in apoE secretion (Fig. 6A) from HMDMs but exerted no significant effect on cellular apoE mRNA or protein levels (not shown). Because PKC\(\gamma\) is not expressed in human macrophages (44), these data indicate a role for PKC\(\alpha/\beta\) and/or PKC\(\beta\) in apoE secretion. Thus, we also explored the PKC\(\beta\) inhibitor, LY379196 (Lilly). Treatment of HMDMs with the PKC\(\beta\) inhibitor LY379196 (10 \(\mu\)M) decreased apoE secretion by 22.9 \(\pm\) 2.3% (\(n = 4\) different donors) (Fig. 6B). Although the concentration of LY379196 used here may potentially target other PKC isoforms, human macrophages often require a higher concentration of pharmacological inhibitors than many cell lines for effect, and LY379196 has been used previously in primary human macrophages at concentrations up to 10 \(\mu\)M (65). To confirm involvement of PKC\(\alpha/\beta\) and/or PKC\(\beta\), HMDMs were incubated with siRNA against either PKC\(\alpha\) or PKC\(\beta\). However, most likely due to the strong sequence homology between these PKC isoforms, siRNAs designed against either PKC\(\alpha\) or PKC\(\beta\) also targeted the opposite isoform. Treatment of HMDMs with PKC\(\alpha/\beta\) siRNAs 228 and 429 resulted in a marked reduction in PKC\(\alpha/\beta\) mRNA (data not shown) and protein levels (64.4 \(\pm\) 8.3 and 71.8 \(\pm\) 2.4%, respectively) (Fig. 6C). Both siRNAs 228 and 429 resulted in an inhibition of apoE secretion (39.1 \(\pm\) 7.4 and 39.2 \(\pm\) 8.0%, respectively) (Fig. 6D). There was a minor decrease in cellular apoE levels (data not shown). In contrast, knockdown of PKC\(\alpha\) (59.7 \(\pm\) 1.5 to 68.7 \(\pm\) 1.3%) in human macrophages (Fig. 6E) had no effect on apoE secretion (Fig. 6F) or cellular apoE levels (data not shown).
Inhibitory peptides specific for PKCε (Fig. 6G), PKCθ, and PKCζ (Fig. 6H) also had no effect on apoE secretion. Taken together, our data strongly support a role for classical isoforms PKCα and -β, but not PKC isoform δ, ε, θ, or ζ, in macrophage-mediated apoE secretion.

**PKC Regulates ApoE Secretion via MARCKS**—To determine potential downstream targets or other signaling proteins involved in regulating PKC-mediated apoE secretion, we investigated proteins known to be involved in PKC-regulated protein secretion in other cells (66–71). HMDMs were treated with PMA, Ro-31-8220, or Gö6976 for 5 min, and the ratios of phosphoprotein/total protein were determined using Western blot analysis. PMA treatment caused a 3.3-fold increase in phosphorylation of ERK44/42 upon PKC inhibition. Last, CypA and HSP90 were unaffected by PKC inhibition. In contrast, lysozyme and fibronectin release were not stimulated by PMA, but their secretion was decreased with PKC inhibition. In contrast, lysozyme and fibronectin release were not stimulated by PMA, but their secretion was decreased with PKC inhibition. Last, CypA and HSP90 were unaffected by PKC inhibition. Thus, PKC modulation demonstrates a degree of specificity for the secretion of different cargos in human macrophages.

**DISCUSSION**

This is the first report of a role for the classical PKCα/β isoforms and MARCKS in regulating apoE secretion from any cell type and the first to demonstrate a specific role for PKC in regulating vesicular protein transport in HMDMs. Importantly, these results also confirm PKC as a signaling target capable of enhancing apoE secretion from HMDMs.

A range of structurally diverse chemical PKC inhibitors, a PKC inhibitory peptide, and PKCα/β isoform siRNA knockdown all inhibited apoE secretion from HMDMs and clearly indicate a role for PKC in this process. Our results show that PKC inhibition directly regulates apoE secretion. PKC inhibition prevented the secretion of recently synthesized [35S]apoE and inhibited transport of apoE-GFP-containing vesicles but did not affect apoE glycoform distribution, suggesting that PKC acts on the transport of apoE after completion of its glycosylation and sialylation in the Golgi and trans-Golgi networks. Levels of cell-associated apoE were minimally affected by PKC
inhibition. This may be due to efficient degradation of unsecreted apoE or the relatively small size of the retained pool relative to cellular apoE, which limits the sensitivity with which small degrees of accumulation could be detected under our experimental conditions.

A role for PKA, PP2B, and phospholipase C signaling pathways in regulation of apoE secretion has only recently been elucidated (16, 27). PKC and PKA share a number of similarities in their effects on apoE secretion. Inhibition of PKA and PKC similarly directly inhibit apoE secretion without materially affecting apoE mRNA or cell apoE protein levels, and both profoundly inhibit the movement of apoE-containing vesicles in macrophages. These results clearly distinguish the effects of PKC from those of PP2B, whose inhibition with cyclosporin A reduced apoE secretion and degradation, resulting in substantial apoE accumulation in the cell. In addition, PP2B inhibition did not affect the transport of apoE-containing vesicles. PKC, however, differs from PKA in that stimulation of PKC activity...
with PMA rapidly increased apoE secretion, whereas stimulation of PKA activity did not (16). These distinctions indicate that PKC may have a unique role in the stimulation of apoE secretion in human macrophages, providing a signaling pathway by which secretion of apoE can be enhanced. Furthermore, the stimulation of apoE secretion by PMA is decreased by both PKA and P2B inhibition (Fig. 5, D and E), suggesting that PKC is likely to be upstream of PKA and P2B in the signaling cascade regulating apoE secretion. Further, chelation of Ca\(^{2\+}\) by BAPTA-AM and blocking Ca\(^{2\+}\) release from the endoplasmic reticulum using the inositol 1,4,5-trisphosphate receptor antagonist 2-APB both decreased PMA-mediated apoE secretion (Fig. 5F), confirming the importance of Ca\(^{2\+}\) in regulating this process.

The stimulation of apoE secretion by PMA implies that classical and novel PKC isoforms, but not atypical isoforms, are involved in stimulated apoE secretion. This is also supported by our data demonstrating that CalpC, Ro-31-8220, Bisl, G66976, LY379196, and PKC inhibitory peptide all inhibited apoE secretion based on their reported specificities (45; 55; 65). The effects of G66976 (inhibitor of classical PKC isoforms) and LY379196 (PKC\(\beta\) inhibitor) on apoE secretion (decrease in apoE secretion) imply that PKC\(\alpha\) and/or PKC\(\beta\) are most likely to regulate apoE secretion in HMDMs because these cells do not express PKC\(\gamma\) (44). The use of pharmacological inhibitors to study cell signaling molecules, such as PKC, can be complicated by off-target effects, particularly at higher concentrations. This problem is reduced, but not eliminated, by the use of multiple inhibitors at the lowest effective concentrations, as we have done in this study. The role of PKC\(\alpha/\beta\) was further confirmed by our siRNA studies. Because both PKC\(\alpha\) and PKC\(\beta\) share \(~80%\) homology, designing specific siRNA oligonucleotides that exclusively target one of the isoforms is technically challenging, and we found that all oligonucleotides, although specifically designed to affect only one isoform, also inhibited the other. Thus, although we cannot be conclusive about the relative importance of PKC\(\alpha\) and PKC\(\beta\) in regulating apoE secretion or the possibility of functional redundancy of the two isoforms, a role for one or both of these isoforms is clear. siRNA knockdown of the novel PKC isoform, PKC\(\delta\), and peptides inhibitory to PKC isoform \(\delta\), \(\theta\), or \(\iota\) had no effect on apoE secretion from human macrophages (Fig. 6, E–H). Future studies using cells from PKC\(\alpha\) or PKC\(\beta\) knock-out mice may be useful to elucidate the relative importance of either of these isoforms in regulating apoE secretion.

The expression and function of specific PKC isoforms varies between cells, tissues, and species (30). For example, PKC\(\alpha\) is expressed in abundance in the human leukemia cell line (HL-60), whereas it has relatively minor expression in rat brain cells (80) and in primary macrophages. Thus, studies using primary human macrophages, although technically challenging, appear to be essential for clarifying PKC and isoform specificity in relation to apoE secretion.

There are very few studies of vesicular transport in primary human macrophages. Here, we transfected primary human macrophages with apoE-GFP to track apoE-containing vesicles. Inhibition of PKC with pharmacological inhibitors arrested apoE-containing vesicles rapidly, identifying the site of action at the vesicular transport level. A number of vesicular transport proteins have been linked to PKC activation in cell systems other than macrophages, including ADP-ribosylation factor, \(\beta^\prime\)-COP, and MARCKS. Our data indicate that the PKC activation or inhibition corresponds directly with the degree of MARCKS phosphorylation (Fig. 7A) and is supported by inhibited apoE secretion by the peptide inhibitor, BIO-11000. MARCKS regulates the hypersecretion of mucus in respiratory diseases, such as asthma and cystic fibrosis (70; 81). Phosphorylation of MARCKS allows movement to the cytoplasm, binding to chaperone HSP70, which engages the cysteine string protein located on the surface of the secretory vesicles containing mucin (69, 82). Because apoE is structurally a mucin, we hypothesize a similar model for apoE secretion, and future research will delineate the exact chaperone molecules and mechanisms through which MARCKS regulates both basal and stimulated apoE secretion.

PKC has effects on the secretion of other proteins released from human macrophages but demonstrates some selectivity. We confirmed that MMP9 secretion from human macrophages is PKC-dependent as previously reported in other cell types (83, 84). Furthermore, consistent with previous reports in macrophage cell lines, PMA did not increase lysozyme secretion (85). However, PMA did not increase fibronectin secretion from primary human macrophages as previously reported in other cell types (86, 87). The effects of PKC activation and inhibition on CHI3L1 secretion from human macrophages observed in this study are very similar to that of apoE secretion, and these findings are novel. A potential role for CHI3L1 has been identified in Gaucher disease and atherosclerosis, where distinct subsets of macrophages express CHI3L1 and its homolog, chitinotri- dase (88). However, the mechanisms through which CHI3L1 mediates these disease processes remain to be elucidated.

PKC plays a role in various disease states, such as bipolar disorders, diabetes, arteriosclerosis, Alzheimer disease, cardiac hypertrophy, and various types of cancer (30, 43, 64). A number of animal models and clinical trials have been conducted with

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**Table 1**

| Secreted protein | PMA | Ro-31-8220 | CalpC |
|-----------------|-----|-----------|-------|
| ApoE            | 61 ± 19\(^a\) | 48 ± 12\(^b\) | 56 ± 6\(^b\) |
| MMP9            | 52 ± 8\(^a\)  | 44 ± 13\(^b\) | 58 ± 8\(^a\) |
| Lysozyme        | 10 ± 9       | 29 ± 5\(^a\)  | 42 ± 20\(^b\) |
| Fibronectin     | -6 ± 15      | 51 ± 15\(^b\) | 67 ± 12\(^b\) |
| CypA            | -12 ± 18     | -29 ± 29      | -38 ± 76     |
| HSP90           | -3 ± 7       | 7 ± 8         | -0.13 ± 54   |

\(^a\)\(^p<0.01, \(^b\)\(^p<0.05\).
pharmacological inhibitors of PKC to treat disorders, such as diabetic peripheral retinopathy (e.g. LY333531/roxubotinastaurin), cancer (e.g. UCN-01 and CGP41251), and autoimmune diseases, such as psoriasis (e.g. AEBO71) (45–48). Based on our results, the effects of such inhibition on protein secretion will vary with the protein cargo and the PKC isoforms affected. The inhibition of apoE secretion by PKC inhibitors could have complex effects on atherosclerosis and immune function.

In summary, this is the first study to identify a role for PKC and MARCKS in regulating both basal and stimulated apoE secretion from primary human macrophages. Pharmacological inhibition of specific PKC isoform(s) and siRNA knockdown of PKCe/β suggest that the classical PKC isoforms are involved in mediating apoE secretion.

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