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Protein kinase C β and δ isoenzymes mediate cholesterol accumulation in PMA-activated macrophages

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Abstract

Previously, we showed that PMA activation of human monocyte-derived macrophages stimulates macropinocytosis (i.e., fluid-phase endocytosis) of LDL and transforms these macrophages into foam cells. The current study aimed to learn which PKC isoenzymes mediate cholesterol accumulation in PMA-activated human macrophages incubated with LDL. Cholesterol accumulation by PMA-activated macrophages incubated with LDL was nearly completely inhibited (>85%) by the pan PKC inhibitors Go6850, Go6983, and RO 32-0432, but only was inhibited about 50% by the classical group PKC inhibitor, Go6976. This indicated that cholesterol accumulation was mediated by both a classical group and some other PKC isoenzyme. PKC β was determined to be the classical group isoenzyme that mediated PMA-stimulated cholesterol accumulation. A pseudosubstrate myristoylated peptide inhibitor of PKC α and β showed partial inhibition (≅50%) of cholesterol accumulation. However, a small molecule inhibitor of PKC α, HBDDE, show minimal inhibition of cholesterol accumulation while a small molecule inhibitor of PKC β, LY333513, could completely account for the inhibition of cholesterol accumulation by the classical group PKC isoenzyme. Thus, our findings show that β and some other PKC isoenzyme, most likely δ, mediate cholesterol accumulation when macropinocytosis of LDL is stimulated in PMA-activated human monocyte-derived macrophages.
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Macrophage foam cell formation is an important process in atherosclerotic plaque development. Uptake and storage of plasma lipoprotein-derived cholesterol within monocyte-derived macrophages affects macrophage functions in ways that influence plaque development and stability. Whether macrophage storage of cholesterol contributes to cholesterol retention within the plaque or facilitates its removal from plaques remains to be determined [1,2]. In either case, macrophage storage of cholesterol promotes macrophage expression of proteases and tissue factor that contribute to plaque rupture and thrombosis, respectively [3–5].

Previously, macrophage foam cell formation was thought to occur only through uptake of modified forms of low density lipoprotein (LDL) such as oxidized or aggregated LDL [2]. Recently, we showed that macrophage foam cell formation can occur through uptake of native LDL in a receptor-independent fashion mediated by fluid-phase macropinocytosis [6–8]. When macrophages are differentiated from human monocytes in human serum, activation of these macrophages with the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), stimulates macrophage uptake of fluid in macropinosomes and any LDL contained in that fluid. With these human macrophages, ^{125}I-LDL uptake is linearly related to LDL concentration, does not show saturation at high LDL concentrations, and cannot be competed by unlabeld LDL.

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Moreover, LDL can be visualized entering macrophages within the fluid-phase of macropinosomes [7].

PKCs are serine–threonine kinases functioning in signal transduction pathways that mediate a variety of cell functions. PKCs are comprised of a number of isoenzymes subclassified into three groups. The classical group of PKCs consist of PKCs α, β, and γ with two isoenzymes of β (β1 and β2) due to alternative splicing of the PKC β gene message. The novel group includes PKCs δ, ε, θ, and η. The atypical group includes PKCs λ, ι, μ, and ζ. Because the atypical group PKCs lack the phorbol ester-binding domain found in classical and novel PKCs, members of this PKC subgroup are not stimulated by PMA and would not be expected to directly mediate cellular actions induced by PMA activation of macrophages.

In this study, we have determined which PKC isoenzymes mediate PMA stimulation of macrophage cholesterol accumulation during incubation with native LDL. Elucidation of which PKCs mediate a specific cellular function can be investigated by the use of small molecule inhibitors that target the ATPase domain of the PKCs, and myristoylated peptides that target the pseudosubstrate binding domain of the PKCs. In addition, immunoblotting of cell extracts can be used to determine which PKC family members are present in the cell under study. Another characteristic of PKCs that can be used to examine PKC functioning in cellular processes is PMA-induced downregulation of PKC enzyme within cells. Some but not all PMA-activatable PKC isoenzymes in a given cell type show downregulation following prolonged PMA stimulation.

Currently, there is great interest to specifically target PKC isoenzymes with small molecule inhibitors, and certain PKC isoenzyme inhibitors are undergoing clinical trials [9]. Determining which PKC isoenzymes mediate human macrophage cholesterol accumulation will be relevant for future attempts to modulate this process with inhibitors as well as to anticipate which PKC isoenzyme inhibitors in current clinical development for other diseases may also affect atherosclerosis disease development.

Materials and methods

PKC inhibitors. PKC pseudosubstrate myristoylated peptide inhibitors for α + β (P-205) and ε (P-223), HBDDE, RO 32-0432, Go6976, and Go6983 were obtained from Biomol. PKC pseudosubstrate myristoylated peptide inhibitors for theta (#539636) and eta (#539602) and Go6850 were obtained from Calbiochem. LY333531 was obtained from Alexis. The non-PKC inhibitors genistein and H89 were obtained from Biomol.

Culture of human monocyte-derived macrophages. Human monocytes were purified with counterflow centrifugal elutriation of mononuclear cells obtained by monocytopheresis of normal human donors. The monocytes were cultured in pooled human AB, heat-inactivated serum (Pel-Freez) as described previously [10] except that 0.4×10^6 monocytes/cm^2 were initially seeded into 12-well (22-mm diameter) culture plates (Plastek C from MatTek). For experiments, two-week-old monocyte-derived macrophage cultures were rinsed 3 times with RPMI-1640 medium and then incubated in triplicate at 37 °C for the indicated times with the indicated additions to RPMI-1640 medium without serum.

Preparation of LDL for use in experiments. Before use, human LDL (Intracel) was dialyzed against 1 liter of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4 °C, then against RPMI-1640 medium (2 changes, 1 L/each change) for 24 h. Dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cut-off). After dialysis, lipoproteins were sterilized by passage through a 0.45-μm (pore-size) low-protein binding filter (Gelman Acrodisc). Aggregated LDL was prepared either by vortexing or by treatment with sphingomyelinase as described previously [11]. LDL concentration is expressed in terms of protein.

Assay of cholesterol and protein contents of macrophages. After incubations, macrophages were rinsed 3-times each with Dulbecco's phosphate-buffered saline (DPBS) plus Mg^{2+}, Ca^{2+}, and 0.2% bovine serum albumin (BSA), and then DPBS plus Mg^{2+} and Ca^{2+}. Macrophages were harvested from wells by scraping into 1 ml distilled water, and then processed as described previously [10]. Lipids were extracted from an aliquot of cell suspension using the Folch method [12]. The cholesterol content of macrophages was determined according to the fluorometric method of Gamble et al. [13]. Macrophage protein content was determined on another aliquot of cell suspension by the method of Lowry et al., using BSA as a standard [14]. Protein contents of cultures generally ranged between 0.2–0.3 mg/well.

SDS–PAGE immunoblotting. To assess PKC isoenzymes, macrophages were lysed with a buffer containing 20 mM Tris–HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM PMSF, and 10 μg/ml leupeptin, then sonicated briefly with a microtip ultrasonic cell disruptor and centrifuged. Equal amounts of protein were subjected to SDS–PAGE and electrophoresed under reducing conditions. Separated proteins were electroblotted onto nitrocellulose membranes (Amersham) using a semi-dry blotter. The membranes were then blocked for 1 h in 150 mM NaCl, 100 mM Tris, pH 7.4, containing 0.1% Tween 20 (TBST). Rabbit polyclonal and mouse monoclonal antibodies against PKC isoenzymes (Santa Cruz) were incubated 3 h with the membrane at a dilution of 1:1000. After three washes with TBST, the membrane was treated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Transduction Laboratories, 1:3000 dilution) in TBST. After three washes, the protein bands were visualized with an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham).

Statistical analysis. All data are presented as the means ± standard error. The means were determined from three culture wells for each data point. The unpaired Student's t test was used to compare significant differences between means. A p value <0.05 was considered significant.

Results

Kinase dependency of PMA-stimulated macrophage cholesterol accumulation

Previously we showed that PMA-stimulated macrophage cholesterol accumulation during incubation with native LDL could be inhibited with broad specificity PKC inhibitors [6]. To learn whether other protein kinase classes functioned in PMA-stimulated cholesterol accumulation we tested the effects of genistein, a pan tyrosine kinase inhibitor, and HA89, a cyclic AMP-dependent protein kinase (PKA) inhibitor, on this process. Neither of these protein kinase inhibitors significantly decreased PMA-stimulated cholesterol accumulation, while the pan PKC inhibitor, Go6850 (also referred to as GF109203X), almost completely prevented PMA-stimulated cholesterol accumulation (Fig. 1).

The inhibitory effect of Go6850 on PMA-stimulated macrophage cholesterol accumulation during incubation

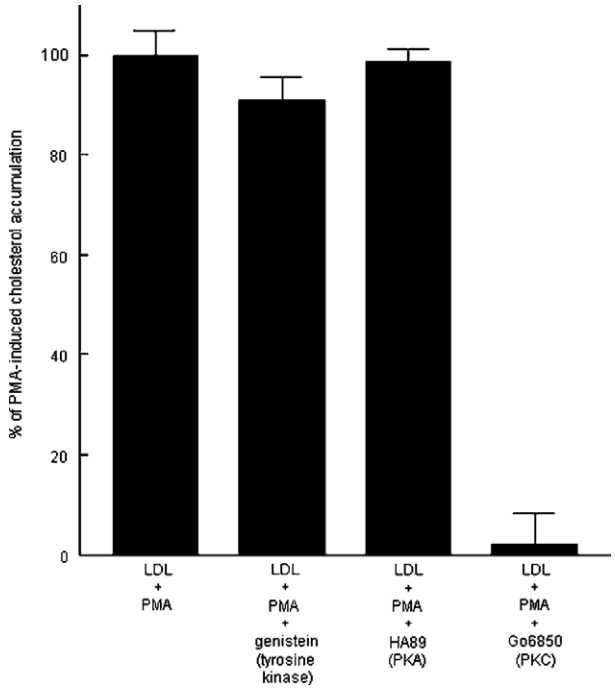

Fig. 1. Role of protein kinases in macrophage cholesterol accumulation. Macrophages were incubated 8 hours with 2 mg/ml LDL and 1 μg/ml PMA with either the tyrosine kinase inhibitor, genistein (25 μg/ml), the cyclic AMP-dependent protein kinase (PKA) inhibitor, HA89 (25 μM), or the protein kinase C (PKC) inhibitor, Go6850 (3 μM). Following incubations, macrophages were rinsed and harvested to measure their protein and cholesterol content. The net cholesterol accumulated by PMA-activated macrophages was considered the control and assigned a value of 100%.

with native LDL was not a non-specific effect on macrophage function. We previously showed that aggregated LDL is taken up into surface-connected compartments by these macrophages through a novel endocytic process, patocytosis [15]. Go6850 did not block cholesterol accumulation when macrophages were incubated 1 day with 100 μg/ml aggregated LDL prepared either by vortexing or by treatment with sphingomyelinase (data not shown).

Western blot analysis of macrophage PKC isoenzymes

An initial assessment of the PKC isoenzymes present in the 2-week-old differentiated human monocyte-derived macrophages was carried out by immunoblot analysis of whole cell lysates (Fig. 2). PKC isoenzymes α, β, δ, ε, θ, ι, ζ, and λ were present, while γ, η, and μ were not detected. Of the PKC isoenzymes present in these macrophages, PKC α, β, and δ showed downregulation with prolonged 48-h incubation of macrophages with PMA. However, while PKC δ and α showed almost complete downregulation, PKC β showed only partial downregulation demonstrated by a visible immunostained band present even after the 48-h pre-treatment with PMA.

PKC β mediated part of PMA-stimulated macrophage cholesterol accumulation

We next utilized an inhibitor of the classical group of PKCs (α, β, and γ) to find out whether PMA-stimulated macrophage cholesterol accumulation could be explained exclusively by this group of PKCs. While pan PKC inhibitors, Go6850 and Go6983 [16], showed almost complete inhibition (>90%) of PMA-stimulated cholesterol accumulation (Table 1 and upper panel of Table 2), an inhibitor of the classical group PKCs, Go6976 [17], showed only partial inhibition of cholesterol accumulation (68% in Experiment 1 under Table 1 and 48% in the upper panel of Table 2). This suggested that PKCs in both the classical and novel groups mediated PMA-stimulated cholesterol accumulation.

To learn which PKCs mediated LDL-induced cholesterol accumulation, we used available myristoylated peptide sequences that bind to unique pseudosubstrate binding sites present within the regulatory domain of some PKCs

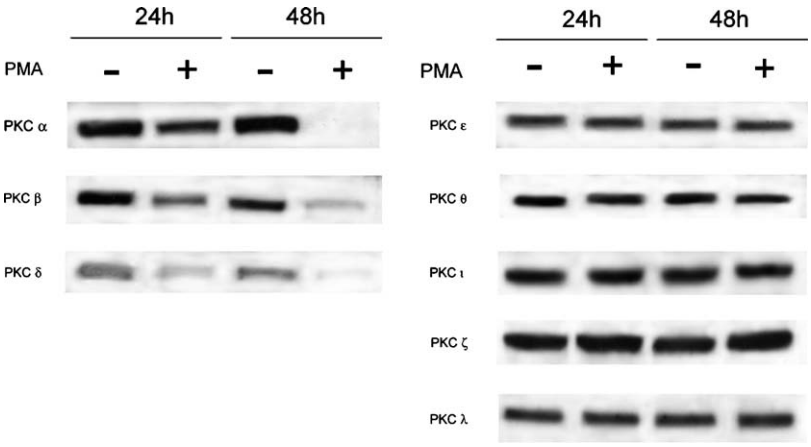

Fig. 2. PMA-induced downregulation of macrophage PKC isoenzymes. Macrophages were incubated 24 or 48 h without or with 1 μg/ml PMA. Then, the macrophages were harvested in lysing buffer and total cell lysates were analyzed for the presence of PKC isoenzymes by Western blot analysis following SDS–PAGE. PKCs γ, η, and μ were not detected. α is alpha; β is beta; δ is delta; ε is epsilon; θ is theta; ι is iota; ζ is zeta; λ is lambda.

Table 1
Effect of PKC inhibitors on PMA-stimulated cholesterol accumulation

Condition	Macrophage total cholesterol (nmoles/mg cell protein)	Inhibition (%)
Experiment 1		
No addition	97 ± 5	—
LDL (2 mg/ml)	99 ± 5	—
LDL + PMA (1 μg/ml)	325 ± 11	—
LDL + PMA + Go6850 (4 μM)	109 ± 1	95
LDL + PMA + Go6976 (4 μM)	171 ± 13	68
Experiment 2		
No addition	74 ± 2	—
LDL (2 mg/ml)	125 ± 6	—
LDL + PMA (1 μg/ml)	470 ± 16	—
LDL + PMA + RO 32-0432 (10 μM)	180 ± 9	73
LDL + PMA + HBDDE (100 μM)	419 ± 37	13

Macrophages were incubated 2 days with the indicated additions. Then, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol content. The percent inhibition of PMA-stimulated cholesterol accumulation compared with no addition is shown.

Table 2
Effect of PMA pre-treatment on PMA-stimulated cholesterol accumulation

Condition	Macrophage total cholesterol (nmoles/mg cell protein)	Inhibition (%)
Pre-treatment without PMA		
No addition	83 ± 5	—
LDL (2 mg/ml)	105 ± 6	—
LDL + PMA (1 μg/ml)	444 ± 9	—
LDL + PMA + Go6850 (5 μM)	120 ± 4	90
LDL + PMA + Go6983 (5 μM)	107 ± 5	93
LDL + PMA + Go6976 (5 μM)	270 ± 6	48
LDL + PMA + LY333531 (1 μM)	244 ± 14	55
Pre-treatment with PMA		
No addition	80 ± 3	—
LDL (2 mg/ml)	133 ± 7	—
LDL + PMA (1 μg/ml)	229 ± 15	—
LDL + PMA + Go6850 (5 μM)	124 ± 5	70
LDL + PMA + Go6983 (5 μM)	100 ± 6	87
LDL + PMA + Go6976 (5 μM)	90 ± 3	93
LDL + PMA + LY333531 (1 μM)	91 ± 3	93

Macrophages were first pre-treated without or with PMA (1 μg/ml) for 2 days and then incubated 1 day with the indicated additions. Following incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol content. The percent inhibition of PMA-stimulated cholesterol accumulation compared with no addition is shown. PMA pre-treatment of macrophages inhibited subsequent PMA-stimulated cholesterol accumulation by 59% compared with macrophages that were not pre-treated with PMA.

[18]. We did not observe inhibition of PMA-stimulated cholesterol accumulation by myristoylated pseudosubstrate peptide inhibitors specific for θ and ε, two novel group PKCs that were present in the macrophages (Table 3). At the same time, a myristoylated pseudosubstrate peptide inhibitor specific for PKC α + β showed partial inhibition of cholesterol accumulation (56%) similar and consistent with the partial inhibition of cholesterol accumulation described above for the classical group inhibitor, Go6976. A myristoylated pseudosubstrate peptide inhibitor specific for PKC η, which was not present in these macrophages, served as a negative control and showed no inhibition.

In another experiment (Experiment 2 in Table 1), RO 32-0432, a pan PKC inhibitor [19], also showed substantial inhibition (73%) of cholesterol accumulation. However, the PKC α and γ inhibitor HBDDE [20] only minimally inhibited (13%) PMA-stimulated cholesterol accumulation showing that these classical group PKCs did not mediate cholesterol accumulation. The finding suggested that PKC β was the PKC isoenzyme of the classical group contributing to PMA-stimulated cholesterol accumulation. To confirm PKC β involvement, we tested the effect on cholesterol accumulation of a PKC β specific small molecule inhibitor, LY333531 [21]. LY333531 inhibited PMA-stimulated cholesterol accumulation by 55% thus accounting for all the inhibitory effect, 48%, observed with the PKC classical group inhibitor, Go6976 (upper panel of Table 2).

Effect of PMA-induced PKC downregulation on PKC inhibitor effect

The above results indicate that PKC β and some other PKC each contributed about 50% of the PMA-stimulated cholesterol accumulation. Pre-treatment of macrophages with PMA for 2 days caused a 59% reduction in cholesterol accumulation compared with macrophages without PMA pre-treatment before their incubation with PMA and LDL (Table 2). Pre-treatment of macrophages with PMA would be expected to downregulate some PKCs. If these PKCs functioned in PMA-stimulated cholesterol accumulation, then this could account for the decreased cholesterol accumulation observed when the PMA-pretreated macrophages were subsequently incubated with PMA and LDL. Following this line of reasoning, we correlated loss of PMA-stimulated cholesterol accumulation with loss of PMA isoenzymes induced by PMA pre-treatment of macrophages. PKC α and δ were completely downregulated by pre-treatment with PMA (Fig. 2). This suggests that PKC δ was the other PKC isoenzyme mediating PMA-stimulated cholesterol accumulation because we showed above that inhibitors of PKC α did not substantially inhibit cholesterol accumulation.

If PKC δ did function in PMA-stimulated macrophage cholesterol accumulation, then following downregulation of this PKC isoenzyme, PMA-stimulated cholesterol accumulation should be more completely inhibited by PKC β inhibitors. This turned out to be the case. We pre-treated macrophages with PMA for 48 h, and during subsequent incubation with PMA and LDL we added either the classical PKC inhibitor, Go6976, or the PKC β inhibitor, LY333531. Following macrophage pre-treatment with PMA, Go6976, and LY333531 nearly completely inhibited subsequent PMA-stimulated cholesterol accumulation

Table 3
Effect of PKC pseudosubstrate peptide inhibitors on PMA-stimulated cholesterol accumulation

Condition	Macrophage total cholesterol (nmoles/mg cell protein)	Inhibition (%)
No addition	91 ± 3	—
LDL (2 mg/ml)	108 ± 4	—
LDL + PMA (1 ug/ml)	221 ± 16	—
LDL + PMA + PKC inhibitor α, β (50 μM)	148 ± 3	56
LDL + PMA + PKC inhibitor θ (50 μM)	207 ± 4	11
LDL + PMA + PKC inhibitor η (50 μM)	235 ± 20	−11
LDL + PMA + PKC inhibitor ε (50 μM)	211 ± 5	8

Macrophages were incubated 8 hours with the indicated additions including the designated myristoylated PKC pseudosubstrate peptide inhibitors. Then, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol content. The percent inhibition of PMA-stimulated cholesterol accumulation compared with no addition is shown. A minus value means that the inhibitor increased PMA-stimulated cholesterol accumulation rather than decreased it. Only the PKC pseudosubstrate peptide inhibitor of α and β showed a statistically significant inhibition of PMA-stimulated cholesterol accumulation. α is alpha; β is beta; θ is theta; η is eta; ε is epsilon.

(lower panel of Table 2). This was in contrast to the approximately 50% inhibition of cholesterol accumulation caused by Go6976 and LY333531 when PKC enzyme activity was not downregulated by pre-treatment of macrophages with PMA (upper panel of Table 2).

Discussion

Macrophage foam cell formation has been thought to occur only by receptor-mediated uptake of modified LDL. We recently showed that PMA-activated macrophages take up native LDL through receptor-independent fluid-phase macropinocytosis and form foam cells due to massive accumulation of LDL-derived cholesterol [6,7]. In this report, we have identified β and some additional PKC isoenzyme, most likely δ, as mediators of PMA-stimulated macrophage cholesterol accumulation. These two PKC isoenzymes may function independently to promote PMA-stimulated cholesterol accumulation. Inhibition of PKC β with either Go6976 or LY333531 decreased PMA-stimulated cholesterol accumulation by about 50%. Inhibition of PKCs with Go6850 and Go6983, pan PKC inhibitors, decreased PMA-stimulated cholesterol accumulation by >90% suggesting that PKC β and δ functioned independently and additively to stimulate cholesterol accumulation. In our earlier studies [6,7], microtubule-dependent and -independent macropinocytosis pathways each mediated about 50% of the LDL uptake and cholesterol accumulation consistent with our finding in this study that two different PKC isoenzymes each mediated about 50% of cholesterol accumulated by the PMA-activated macrophages.

Previously, we observed that cholesterol accumulation induced by PMA-activation of macrophages incubated with LDL continued over 2 days and did not substantially downregulate [6]. PMA treatment of cells is known to differentially downregulate various PKC isoenzymes due to an increased rate of PKC degradation [22]. While PKC δ was completely downregulated by 2 days of PMA treatment, PKC β was still present albeit at a much reduced level. This suggests that these two PKC isoenzymes show a temporal difference in PMA-stimulated macrophage cholesterol accumulation with PKC β function continuing after δ function has ceased due to PMA-induced downregulation of the δ isoenzyme. This was confirmed by the finding that PMA-stimulated cholesterol accumulation was completely dependent on the β isoenzyme following PMA pre-treatment of macrophages for 2 days.

Stimulation of macropinocytosis depends on many signaling components including GTPases. One pathway for PKC-mediated induction of macropinocytosis involves Ras, Rac1, and Cdc42 GTPases that promote PAK1 activation and induction of the membrane ruffling that functions in macropinocytosis [23–27]. PKC β activation has been shown to lead to Ras and Rac1 activation which links our findings with the downstream signaling elements that function in macropinocytosis [28,29]. Macropinocytosis also depends on phosphatidylinositol-3′ kinase which functions in the formation of macropinosomes in murine bone marrow-derived macrophages, and in the maintenance, maturation, and translocation of macropinosomes in a macrophage cell line [30,31]. In this regard, phosphatidylinositol-3′ kinase associates with PKC δ in cells suggesting that PKC δ may affect phosphatidylinositol-3′ kinase function [32].

PKC δ and β have been reported to associate with actin consistent with these PKC isoenzymes functioning in macropinocytosis, a process dependent on reorganization of the actin cytoskeleton [33,34]. Actin stress fibers within the cytoplasm must be disassembled, and actin must be redistributed to the plasma membrane to support the formation of plasma membrane ruffles. During macropinocytosis, plasma membrane ruffles fold to fuse with the plasma membrane and form vacuoles that enclose droplets of extracellular medium. PKC-induced Src tyrosine kinase activation can induce this type of actin reorganization and trigger macropinocytosis [35,36]. PKC δ functions in PMA-induced Src activation by forming complexes with Src during Src activation [37].

PKC β mediates macropinocytosis in other cell systems. Adenovirus stimulates PKC-dependent macropinocytosis in HeLa cervical epithelial carcinoma cells [38], and this

PKC-dependent stimulation can be blocked by a pseudosubstrate PKC α + β inhibitor similar to which we used here to inhibit PMA-stimulated cholesterol accumulation. Also, macrophage uptake of apoptotic cells occurs by macropinocytosis and depends on PKC β function [39,40]. Thus, our finding that PKC β mediates macropinocytosis in human monocyte-derived macrophages further implicate this PKC isoenzymes in a general PKC-dependent macropinocytosis signaling mechanism.

How PKC β and δ are activated resulting in macropinocytosis in human monocyte-derived macrophages remains to be determined. However, it is relevant that monocyte PKC β is activated by elevated glucose levels [41], a possible mechanistic link between the accelerated atherosclerosis of patients with diabetes and macrophage cholesterol accumulation within atherosclerotic plaques.

In conclusion, our findings show that β and δ protein kinase isoenzymes are signaling components that mediate macropinocytosis of LDL and foam cell transformation of human monocyte-derived macrophages, a process that we show here can be targeted with specific inhibitors of PKC isoenzymes.

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