G-Protein-Mediated Signaling in Cholesterol-Enriched Arterial Smooth Muscle Cells. 2. Role of Protein Kinase C-δ in the Regulation of Eicosanoid Production[†]

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ABSTRACT: PGI_2 generation by the vessel wall is an agonist for cyclic-AMP-dependent cholesteryl ester hydrolysis. The process of enhanced PGI_2 synthesis is stimulated, in part, by G-protein-coupled receptor ligands. Cellular cholesterol enrichment has been hypothesized to alter G-protein-mediated PGI_2 synthesis. In the studies reported herein, cells generated PGI_2 in response to AlF_4^-, GTPγS, and ATP in a dose-dependent manner. G-protein agonists stimulated eicosanoid production principally by activating phospholipase A_2, but not phospholipase C. This is in contrast to PDGF, which stimulated phospholipase A_2 and PLCγ activities. Gαi subunits mediate G-protein agonist-induced PGI_2 synthesis, since ATP- and PDGF-induced PGI_2 synthesis was inhibited by pertussis toxin. Although cholesterol enrichment reduced arachidonic acid- and PDGF-induced PGI_2 synthesis, cholesterol enrichment enhanced PGI_2 release in response to AlF_4^-, GTPγS, and ATP. The enhancement of PGI_2 release in cholesterol-enriched cells was augmented by mevalonate, which inhibits the ability of cholesterol enrichment to reduce membrane-associated G-protein subunits. Since cholesterol enrichment inhibited PDGF and AlF_4^--induced MAP kinase activity [Pomerantz, K., Lander, H. M., Summers, B., Robishaw, J. D., Balcueva, E. A., & Hajjar, D. P. (1997) *Biochemistry 36*, 9523−9531] (the major mechanism by which phospholipase A_2 is activated), these results suggest that cholesterol enrichment induces other alternative signaling pathways leading to phospholipase A_2 activation. A PKC-dependent pathway is described herein that is involved in enhanced eicosanoid production in cholesterol-enriched cells. This conclusion is supported by two observations: (1) G-protein-linked PGI_2 production is inhibited by calphostin, and (2) cholesterol enrichment augments the specific translocation of the δ-isoform of PKC from the cytosol to the plasma membrane following treatment of cells with phorbol ester. These data support the concept that, in cells possessing normal levels of cholesterol, MAP-kinase-dependent pathways mediate eicosanoid synthesis in response to G-protein activation; however, under conditions of high cellular cholesterol levels, augmented G-protein-linked eicosanoid production results from enhanced PKCδ activity.

INTRODUCTION

Cytokines regulate numerous aspects of arterial smooth muscle cell function. The mitogenic effects of these agonists on smooth muscle cells can be modulated by endogenously synthesized eicosanoids, such as prostacyclin (PGI_2)[1] and PGE_2 (Libby et al., 1988; Pomerantz & Hajjar, 1989). In addition, the control of intracellular cholesterol trafficking by growth factors can also be mediated by PGI_2. This eicosanoid stimulates lysosomal and cytoplasmic cholesteryl ester hydrolase (ACEH, NCEH) activities through cyclic AMP (cAMP) and the cAMP-dependent protein kinase (Hajjar et al., 1982; Hajjar, 1986).

PGI_2 generation is dependent upon signal transduction pathways leading to MAP-kinase-dependent activation of phospholipase A_2 via receptor tyrosine kinases, such as the PDGF receptor (Schlessinger, 1995), or through receptors coupled to heterotrimeric G-proteins, such as thrombin and ATP (Dubyak & Elmoatassim, 1993). Phospholipase A_2 is activated via phosphorylation by PKC-α, PKC-δ, or PKC-ζ (Visnjic et al., 1995; Church et al., 1993), resulting in the stimulation of eicosanoid synthesis (Davies et al., 1984; Church et al., 1993). Receptor tyrosine kinases can activate PLCγ through association of their SH-2 domains, while G-protein-coupled receptors activate PLCβ-isozymes by association with free G-protein α subunits. By either pathway, arachidonic acid liberated from cellular phospholipids is metabolized to PGI_2 by PGI_2 synthase.

The observation that accumulation of cholesterol in arterial cells is accompanied by decreased PGI_2 production (Serneri et al., 1986) raises the possibility that decreased second messenger synthesis by cholesterol-enriched cells may be due to alterations in signal transduction pathways. In previous studies, we found that cholesterol-enriched cells display reduced PGI_2-synthetic capacity due to (1) inhibition of arachidonic acid release by linoleate derived from LDL−CE, (2) inhibition of calcium-dependent phospholipase A_2 activity, (3) decreased expression of PGH synthases-1, and (4) reduced transcriptional activation of PGHS-2 in response to PDGF (Pomerantz et al., 1993; Pomerantz & Hajjar, 1989a). We reasoned that the latter may be due in part to reduced membrane expression of p21ras and reduced PDGF-induced MAP-kinase activity (Pomerantz et al., 1997). However, G-protein-coupled receptor ligands are also capable of eliciting eicosanoid synthesis.

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[1] Abbreviations used: CE, cholesteryl ester; cLDL, cationized LDL; LDL, low-density lipoprotein; PDGF, platelet-derived growth factor; PGI_2, prostaglandin I_2, prostacyclin; PI, phosphatidylinositol; PKC, protein kinase C; PL, phospholipase.

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We have recently demonstrated that cholesterol enrichment reduces the membrane expression of heterotrimeric G-proteins as a result of reduced isoprenylation; this effect can be mimicked by a variety of sterols and attenuated by cotreatment with mevalonate (Pomerantz et al., 1997). Here, we examine the mechanisms by which G-protein-agonists stimulate eicosanoid synthesis in smooth muscle cells and assess the mechanism by which cholesterol enrichment alters this process.

EXPERIMENTAL PROCEDURES

Reagents. Antibodies directed against phospholipase C-γ and 14-3-3ζ were purchased from Upstate Biotechnology (Lake Placid, NY). (*E*)-6-(Bromomethylene)tetrahydro-3-(1-napthalenyl)-2*H*-pyran-2-one (haloenol lactone suicide substrate, HELSS) was purchased from Cal-Biochem (San Diego, CA). Antibodies directed against PKC-β, PKC-δ, and PKC-ζ were purchased from Santa Cruz Technology (Santa Cruz, CA). [^{3}H]-6-Keto-PGF$_{1\alpha}$ and [^{3}H]arachidonic acid were purchased from Dupont/New England Nuclear (Boston, MA).

Preparation of Plasma LDL and cLDL. LDL (1.019−1.063 g/mL) was isolated by preparative ultracentrifugation of pooled donor human plasma and cationized using *N*,*N*-dimethyl-1,3-propanediamine (Kodak Chemicals, Rochester, NY) as adduct and 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide−HCl (Aldrich Chemicals, Milwaukee, WI) as catalyst at pH 6.5 (Pomerantz & Hajjar, 1989b). Both native and cationized LDL (cLDL) contained less than 1 nmol of tetramethoxypropane equivalents/mg protein (Kraemer et al., 1993).

Isolation and Culture of Aortic Smooth Muscle Cells. Cells were propagated from explants of rabbit or rat thoracic aorta and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Logan, UT) and 1% penicillin/streptomycin/amphotericin (v/v/v) in an atmosphere of 5% CO_2 in air at 37 °C (Pomerantz et al., 1984). Cells were identified as smooth muscle by their hill-and-valley morphology at confluence and by positive staining for α-actin. Cells were cholesterol-enriched cells by incubation with cLDL (75 μg protein/mL) in DMEM containing 10% FBS for 7 days as previously described (Pomerantz & Hajjar, 1989b).

Radioimmunoassay for 6-Keto-PGF$_{1\alpha}$. The radioimmunoassay for 6-keto-PGF$_{1\alpha}$, the stable hydrolysis product of PGI$_2$, has been previously described by our laboratory (Pomerantz & Hajjar, 1989b). Binding of unknowns was compared to concurrently derived standard curves (6.2−1000 pg of 6-keto-PGF$_{1\alpha}$/0.1 mL). The data are expressed as ng/mg cell protein.

Phospholipase A$_2$ Activity. Cells were labeled with [^{3}H]-arachidonic acid and then exposed to agonists in the presence of 0.1% fatty acid-free bovine serum albumin (Pomerantz & Hajjar, 1989b). Phospholipase A$_2$ activity is expressed as dpm free fatty acid released/mg protein/15 min.

PLC Activity. The measurement of PLC activity was measured by the accumulation of polyinositol phosphates. Normal and cholesterol-enriched cells were labeled with 10 μCi/mL (2.0 mL/plate) *myo*-2-[^{3}H]inositol for 72 h in M-199 containing 2% fetal bovine serum. Cells were washed three times in PBS containing 0.2% BSA and twice in PBS and then incubated for 5 min in PBS containing 10 mM $LiCl_2$. Cells were then exposed to agonists and incubated at 37 °C for 3 min. After removal of the supernatant, 1.0 mL of 15% (w/v) TCA was added and the mixture incubated for 1 h at 4 °C. Cells were then scraped into TCA, transferred to a 2.0 mL Eppendorf tube, and spun at 12000*g* for 2.0 min. The deproteinated supernatant was then lipid-extracted in water-saturated diethyl ether. After addition of 5 μL of 50 mM mannitol (carrier), the sample was lyophilized and reconstituted into 70 μL of distilled water; polyphosphoinositides in a 20 μL aliquot were separated and quantified by HPLC on a Whatman Partisil 10 SAX column (25 cm × 0.46 cm) with a precolumn containing a pellicular anion-exchange resin using a 0.01−0.52 M ammonium phosphate gradient (pH 3.8, buffered with H_3PO_4) (Sakuma et al., 1988). Radioactivity was quantified using an INUS β-RAM in-line radioactivity monitor. The identity of each peak was determined by comigration with labeled standards.

Phospholipase C-γ Phosphorylation. Phosphorylation, hence, activation, of PKCγ was performed by methods previously described with minor modifications (Margolis et al., 1989; Morrison et al., 1990). Briefly, cells grown in 60 mm plates were extracted into 200 μL of RIPA buffer (150 mM NaCl, 50 mM Tris pH, 7.4, 5 mM EDTA, 1% Triton X-100, 0.4% sodium deoxycholate, 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1.0 mM sodium orthovanadate) for 20 min at 4 °C. Aliquots containing 400 μg of cell protein were incubated with anti-PLCγ antibody (1:300 dilution), followed by precipitation using Protein G sepharose. After boiling in Laemmli buffer containing β-mercaptoethanol, samples were subjected to SDS/PAGE (7.0%), transferred to nitrocellulose, and immunoblotted using antibodies directed against phosphotyrosine (4G10). Phosphorylated PLC-γ (MW 190 kD) was visualized using ECL.

Lactate Dehydrogenase Content. Lactate dehydrogenase was measured using a kit (Sigma), procedure 500, and modified for use in cell culture. The standard curve using pyruvic acid was adjusted to a total volume of 1.065 mL using cytoplasmic buffer. For unknowns, 1.0 mL of pyruvate substrate was added to NADH, followed by addition of 65 μL of 500 μL of total cell sonicate. After incubation at 37 °C for 30 min and addition of 1.0 mL of Sigma color reagent, samples were incubated at 25 °C for 20 min. After addition of 2.0 mL of 2.0 N NaOH, samples were incubated at 25 °C for 15 min, followed by optical measurements at OD_{525}. Activity (BB-Units) was calculated from the standard curve as described by the manufacturer.

N-Acetyl-α-glucosaminidase Content. *N*-acetyl-α-glucosaminidase was measured by quantitation of the generation of 4-methylumbelliferone from the hydrolysis of 4-methylumbelliferyl-*N*-acetyl-α-D-glucosaminide by cell sonicates or cell supernatants. The standard (4-methylumbelliferone) was stored as a stock solution in dry methoxyethanol (1 mM). A dilution series ranging from 1.0 to 0.01 mM in log 3 increments was prepared in dry methoxyethanol and stored at 4 °C. For each assay, working solutions of the standard curve were prepared by adding equal volumes of each standard dilution into "working solution" (10 mM sodium citrate, 0.2% Triton X-100, pH 4.5, final$^-$ concentration); 0.1 mL of each working standard was then added to 0.1 mL of cytoplasmic buffer. Blanks consisted of 0.1 mL of substrate buffer without substrate and 0.1 mL of cell cytoplasmic buffer. Unknowns were prepared from either cell supernatants or cells washed twice in ice-cold PBS,

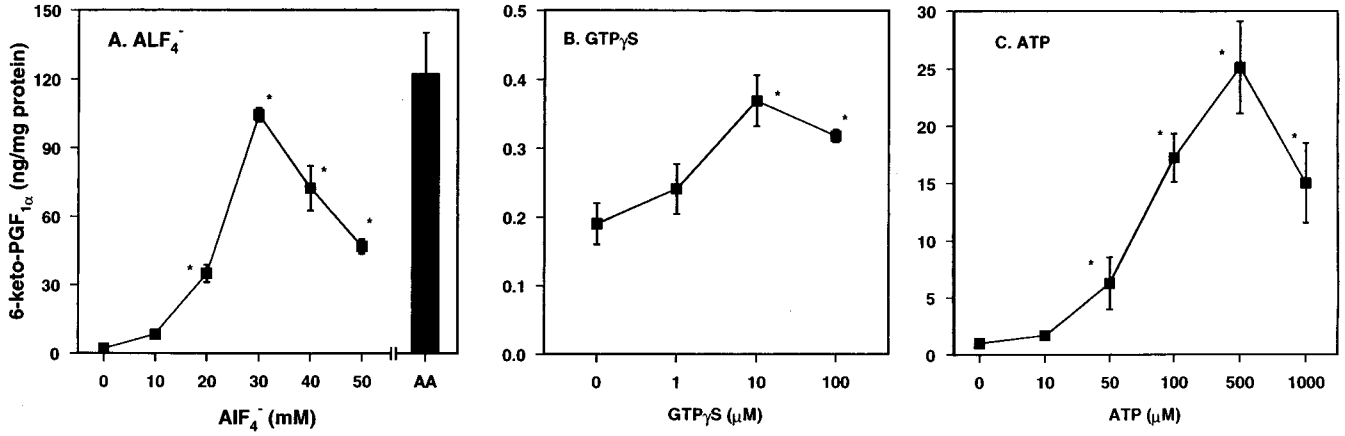

FIGURE 1: Stimulation of PGI_2 release in rabbit aortic smooth muscle cells by G-protein agonists. Cells grown to confluent density were washed in PBS at 4 °C and then incubated in PBS in the presence of AlF_4^- (10−50 mM) or arachidonic acid (5.0 μM) (**A**). Parallel cultures were exposed to "cytoplasmic buffer" (20 mM NaCl, 0.5 mM $MgCl_2$, 102 mM KCl, 25 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 10 mM HEPES, 1.0 mM EGTA, 0.46 mM $CaCl_2$, pH 7.2) containing saponin (12.5 μg/mL) in the presence of GTPγS (0−100 μM) for 15 min at 37 °C (B). These conditions allowed for 60−80% of total LDH release with undetectable *N*-acetyl-α-glucosamidase release (demonstrating selective cell membrane permeabilization without lysosomal permeabilization. Cells were also exposed to PBS containing ATP (0−1000 μM) (C) for 15 min at 37 °C. Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay and normalized to cell protein. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$). Each experiment was performed twice and each treatment performed in triplicate.

scraped, and sonicated in 1.0 mL of "cytoplasmic" buffer. Aliquots (100 μL) were incubated with 100 μL of substrate in a stock solution of 10 mM in dry methoxyethanol. After incubation for 1 h at 37 °C, the reaction was stopped by the addition of 2.0 mL of stopping solution (50 mM glycine, 5 mM EDTA, pH 10.4), and the fluorescence (E_{365} mu/F_{460} mu) of a 200 μL aliquot was read. Activity was calculated as nmoles of umbelliferone/mg of protein.

Cell Permeabilization. For agonist studies using GTPγS, cells were permeabilized using 20 mM NaCl, 0.5 mM $MgCl_2$, 102 mM KCl, 25 mM $NaHCO_3$, 0.96 mM NaH_2PO_4, 10 mM HEPES, 1.0 mM EGTA, and 0.46 mM $CaCl_2$, pH 7.2 (cytoplasmic buffer), containing saponin for 15 min at 37 °C. Preliminary studies were performed to determine the optimal saponin concentration that would allow for cell permeation (as shown by LDH release) without altering either lysosomal permeation (as shown by *N*-acetyl-α-glucosaminidase release) or basal 6-keto-$PGF_{1\alpha}$ production. Saponin promoted little LDH release or 6-keto-$PGF_{1\alpha}$ production at concentrations <10 μg/mL and was linear between 10 and 20 μg/mL, which resulted in 60−80% of maximal LDH release and 6-keto-$PGF_{1\alpha}$ production at 20 μg/mL. *N*-Acetyl-α-glucosaminidase release was undetectable at saponin concentrations of 20 μg/mL. On the basis of these observations, a saponin concentration of 12.5 μg/mL was used, which allowed for approximately 20% of maximal 6-keto-$PGF_{1\alpha}$.

PKC Activity. PKC activity was measured as the translocation of PKC-α and PKC-δ isoforms of PKC from cytosol to membrane following exposure to agonists (Ha & Exton, 1993). Smooth muscle cells contained much less PKC-ζ; PKC-β or PKC-ϵ was undetectable by Western blot (data not shown). PKC-θ is undetectable in smooth muscle cells (Leng et al., 1996); the presence of PKC-η was not evaluated. For each experiment, cells grown in 60 cm^2 dishes were exposed to media alone or media containing PMA (10^{-7} M) for 15 min. Cells were washed and then scraped into 200 μL of homogenization buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 10 mM EGTA, 20 mM β-mercaptoethanol, 0.25 M sucrose, 10 μM leupeptin, 10 μM PMSF, and 0.5% Triton X-100. Cells were then homogenized by passing 17 times through a precooled Dounce homogenizer. Cell fractionations were then performed as previously described (Pomerantz et al., 1997). Western analysis was performed on duplicate 25 μg aliquots of purified membrane and cytosolic fractions (Pomerantz et al., 1993). Primary antibodies were used at a dilution of 1:300, followed by detection using ECL.

RESULTS

Stimulation of PGI_2 Release in Arterial Smooth Muscle Cells by G-Protein-Specific Agonists . To first determine if G-protein stimulation results in eicosanoid synthesis by smooth muscle cells, experiments were performed to determine if G-protein-specific agonists stimulate eicosanoid metabolism. Cells produced significant amounts of PGI_2 in response to AlF_4^- (Figure 1A), GTPγS (Figure 1B), or ATP (a p2y receptor agonist) (Figure 1C). These data demonstrate that G-protein activation triggers eicosanoid production in arterial smooth muscle cells.

Mediation of PGI_2 Release by Gαi Subunits. To determine the role of Gαi subunits in mediating PGI_2 production, cells were incubated with pertussis toxin (100 ng/mL) for 4 h prior to exposure to media alone or media containing ATP (100 μM). Pertussis toxin inhibited PGI_2 synthesis under basal conditions and in response in response to ATP (Figure 2). Thus, eicosanoid synthesis following exposure to these agonists is, in part, pertussis toxin-sensitive, implicating Gαi subunits in mediating eicosanoid generation in response to G-protein-coupled receptor activation.

Mediation of G-Protein-Coupled Eicosanoid Production by Arterial Smooth Muscle Cells Phospholipase A_2, but Not Phospholipase C. We next examined the relative roles of phospholipases A_2 and C in mediating G-protein-coupled phospholipase activation. Cells incorporated 2-[^{3}H]-*myo*-inositol into inositol 1-phosphate (Ins-1P), inositol 2-phosphate (Ins-2P), inositol 4-phosphate (Ins-4P), inositol 1,4-bisphosphate (Ins-1,4-bis-P), and inositol 1,4,5-trisphosphate (Ins-1,4,5,-tris-P) (Figure 3A). AlF_4^- (30 mM) did not promote significant accumulation of Ins-1,4,5-tris-P relative

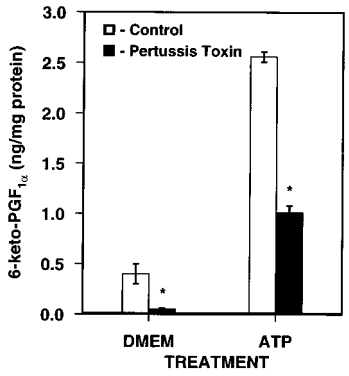

FIGURE 2: Mediation of PGI_2 production in arterial smooth muscle cells by Gαi subunits. Arterial smooth muscle cells were treated with pertussis toxin (100 ng/mL) for 6 h. Cells were then exposed to media alone (CTL) or media containing ATP (100 μM) for 15 min at 37 °C. Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay and normalized to cell protein. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$).

to untreated cells, while PDGF enhanced accumulation of Ins-1P 130% and Ins-1,4-bis-P and Ins-1,4,5-tris-P up to 300% (Figure 3B). Thus, PDGF, but not AlF_4^-, significantly stimulated PLC activity. However, AlF_4^- (30 mM) increased the release of [^{3}H]arachidonic acid by prelabeled cells (Figure 3C). These data support the conclusion that G-protein stimulation of eicosanoid production occurs principally through activation of phospholipase A_2, but not PLC.

Augmentation of PGI_2 Synthesis in Response to G-Protein Activation by Cholesterol Enrichment Role of Pertussis Toxin-Insensitive G-Proteins. Experiments were performed to assess the effects of cholesterol enrichment on PGI_2 synthesis in response to G-protein agonists. Normal and cholesterol-enriched cells were exposed to arachidonic acid, ATP, AlF_4^- and GTPγS (Figure 4). Arachidonic acid-induced PGI_2 production was reduced following cholesterol enrichment, due to reduced expression of PGH synthases-1 and -2 (Pomerantz et al. 1993; Pomerantz & Hajjar, 1989b). However, cholesterol enrichment enhanced ATP-induced PGI_2 release. Importantly, cholesterol enrichment also enhanced PGI_2 generation in response to AlF_4^- and GTPγS, indicating that the enhancement of eicosanoid production by cholesterol-enriched cells in response to G-protein agonists occurs distal to the ATP receptor.

Gαi subunits have been implicated in activation of phospholipases A_2 and C (Marrero et al., 1994). Since cholesterol enrichment reduced Gαi subunits, but augmented eicosanoid synthesis in response to G-protein receptor ligands, these results suggest that cholesterol enrichment may augment pertussis toxin-insensitive pathways that activate PLA_2 or PLC. To test this hypothesis, cholesterol-enriched cells prelabeled with [^{3}H]arachidonic acid were pretreated with pertussis toxin for 6 h prior to exposure to media alone or AlF_4^-. Pertussis toxin did not alter basal [^{3}H]arachidonic acid release (1510 ± 20 vs. 1840 ± 40 dpm/mg protein, CTL vs. pertussis toxin, p = ns, $n = 2$). Pertussis toxin treatment did enhance [^{3}H]arachidonic acid release in response to AlF_4^- (3610 ± 180 vs. 5170 ± 200 dpm/mg protein, CTL vs. pertussis toxin, $p < 0.05$, $n = 2$), suggesting that a pertussis toxin-insensitive pathway enhances eicosanoid generation in response to generalized heterotrimeric G-protein activation. Next, we examined the relative contribution of PLA_2 and PLC in mediating heterotrimeric G-protein-mediated eicosanoid production following cholesterol enrichment. To this end, normal and cholesterol-enriched smooth muscle cells were exposed to media alone, ATP (75 μM), or PDGF (10 ng/mL) for 5 min. The content of phosphorylated PLC-γ was then determined by immunoprecipitation of PLC-γ followed by Western blot of phosphotyrosine (Figure 5). Phosphorylated PLCγ was undetectable in cells not treated with agonists or ATP. PDGF-induced phosphorylation of PLC-γ that was similar between control and cholesterol-enriched cells (Figure 5). In addition, enhanced eicosanoid production in cholesterol-enriched cells was not due to enhanced PLC activity since the generation of Ins 1,4,5-trisphosphate in response to AlF_4^- was not altered following cholesterol enrichment relative to controls (data not shown). These results suggest that cholesterol enrichment enhances G-protein-dependent phospholipase A_2 activity (and subsequent eicosanoid synthesis) in a pertussis toxin-insensitive manner.

Enhancement of Eicosanoid Output in Cholesterol-Enriched Smooth Muscle Cells by Mevalonate. Cholesterol enrichment has been shown to reduce G-protein-mediated signaling by inhibiting the prenylation of low molecular weight G-proteins and heterotrimeric G-protein subunits; the inhibitory effect of cholesterol enrichment on this process could be attenuated by mevalonate (Pomerantz et al., 1997). To test the hypothesis that mevalonate could normalize G-protein-linked eicosanoid metabolism in cholesterol-enriched cells, cells were exposed to cLDL in the presence and absence of mevalonate. Mevalonic acid did not alter basal PGI_2 release in control or cholesterol-enriched cells (data now shown). In addition, mevalonate did not significantly alter PGI_2 release in control cells challenged with AlF_4^-. However, mevelonate significantly elevated AlF_4^--induced PGI_2 synthesis in cholesterol-enriched cells (Figure 6). We interpret this data to indicate that cholesterol enrichment induces a pathway that couples G-proteins to phospholipase A_2. Mevalonate add-back experiments to cholesterol-enriched cells thus allows for normalization of heterotrimeric G-protein content, resulting in further enhancement of G-protein-coupled eicosanoid synthesis.

Enhanced G-Protein-Mediated Eicosanoid Production by Cholesterol-Enriched Cells Caused by Enhanced Protein Kinase C Activity. Phospholipase A_2 is activated by MAP-kinases (Sa et al., 1995) and protein kinase C isoforms (Visnjic et al., 1995; Church et al., 1993; Wijkander & Sundler, 1994). Since cholesterol enrichment inhibited MAP-kinase activity in response to PDGF and AlF_4^- (Pomerantz et al. 1997), we tested the hypothesis that enhanced G-protein-mediated eicosanoid production may be due to enhanced PKC activity. Accordingly, we examined the effect of calphostin C (a specific PKC inhibitor) on AlF_4^--induced PGI_2 production. Normal and cholesterol-enriched smooth muscle cells were exposed to calphostin C (100 nM) for 30 min at 37 °C. Cells were then exposed to AlF_4^- (30 mM) for 30 min at 37 °C. Calphostin C did not significantly alter AlF_4^--induced PGI_2 production in control cells (1.5 ± 0.03 vs. 1.5 ± 0.02 ng/mg protein). However, Calphostin C significantly reduced PGI_2 production in cholesterol-enriched cells by 25% (2.1 ± 0.04 vs 1.5 ± 0.02 ng/mg protein, $p < 0.05$). These data support the hypothesis that enhanced G-protein-mediated eicosanoid synthesis in cholesterol-enriched cells is due to enhanced PKC activity.

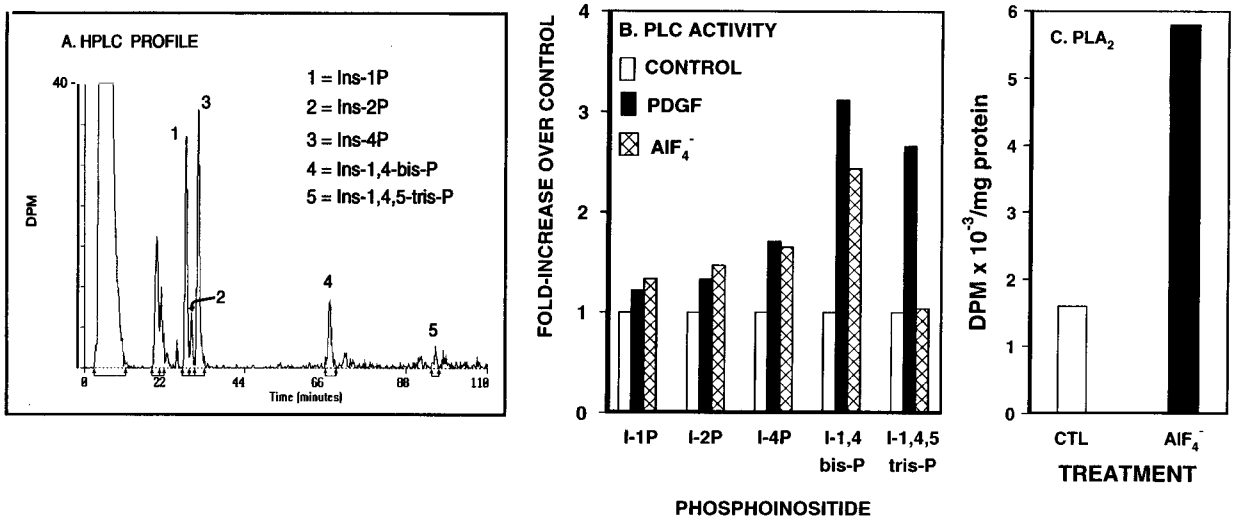

FIGURE 3: PGI_2 production by G-protein agonists: role of phospholipase C and A_2. (A) HPLC profile of inositol phosphates generated from PDGF-stimulated arterial smooth muscle cells. Smooth muscle cells were incubated with [^{3}H]myoinositol for 3 days. Cells were then treated with lithium chloride for 30 min prior to PDGF (2.0 ng/mL) for 15 min at 37 °C. Inositol phosphates were then purified by extraction and quantified by radio-HPLC, as described in the Experimental Procedures. (B) Effect of PDGF and AlF_4^- on polyphosphoinositide synthesis. Cells exposed to *myo*-[^{3}H]inositol (2.0 μCi/plate) and then lithium chloride (10 mM) for 5 min were incubated with either PDGF (2.0 ng/mL) or AlF_4^- (30 mM) for 15 min at 37 °C. Polyphosphoinositides were quantified as described above. PDGF promoted generation of all phosphoinositides, including Ins-1,4,5-tris-P, while AlF_4^- did not promote Ins-1,4,5-tris-P accumulation. This is representative of two experiments, each treatment performed in duplicate. (C) AlF_4^- induction of phospholipase A_2 activity. Cells were incubated overnight with 2.0 μCi/mL with [^{3}H]arachidonic acid. Cells were then exposed to AlF_4^- (30 mM) for 30 min in the presence of 0.1% fatty acid-free bovine serum albumin. This is representative of two experiments, each treatment performed in duplicate (* = $p < 0.05$).

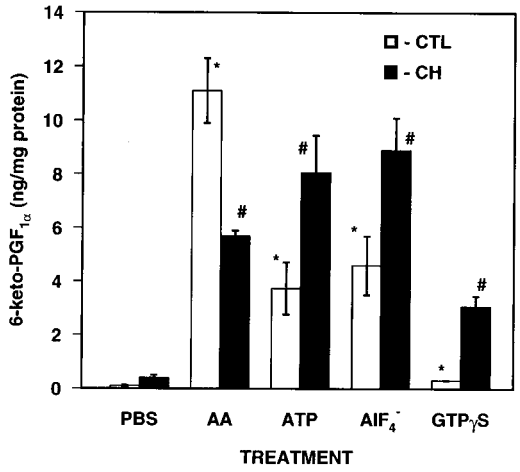

FIGURE 4: Augmentation of G-protein-mediated eicosanoid release by cholesterol enrichment. (A) G-protein-linked PGI_2 release. Control and cholesterol-enriched rabbit cells were exposed to PBS, PBS containing arachidonic acid (AA, 5 μM), ATP (100 μM), AlF_4^- (30 mM), or cytoplasmic buffer containing saponin (12.5 μg/mL) and GTPγS for 30 min at 37 °C. Supernatants from each experiment were assayed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay. Data are expressed as ng of 6-keto-$PGF_{1\alpha}$/mg of protein (mean ± SEM, * = $p < 0.05$, DMEM vs. DMEM plus agonist, # = $p < 0.05$ CTL vs cholesterol-enriched cells). This is representative of two experiments, each treatment performed in triplicate.

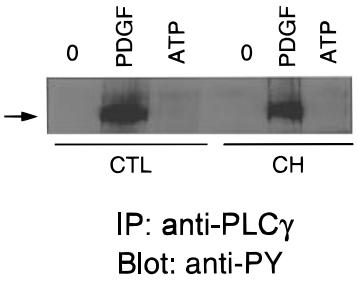

FIGURE 5: Influence of cholesterol enrichment on ATP-mediated activation of PLC-γ. Control and cholesterol-enriched rabbit cells were exposed to media alone (0) or media containing either PDGF (10 ng/mL) or ATP (75 μM) for 5 min. Phosphorylated PLCγ (190 kd, denoted by arrow) was measured by Western analysis using antibodies directed against phosphotyrosine following immunoprecipitation using antibodies directed against PLCγ).

Enhancement of Phorbol Ester-Induced PKC-δ Translocation by Cholesterol Enrichment. To confirm that enhanced G-protein-mediated eicosanoid production in cholesterol enriched cells was due to enhanced PKC activity, the effect of cholesterol enrichment on the membrane and cytosolic content of PKC-isoforms was measured by Western analysis under basal conditions and after exposure of cells to PMA, which promotes translocation of specific PKC isoforms from the cytosol to the membrane. The profile of PKC isoforms contained in rabbit aortic smooth muscle cells was similar to that previously reported (i.e., PKC-α = PKC-δ > PKC-ζ > PKC-ε (Leng et al., 1996; Assender et al., 1995; Ali et al., 1994)). Cholesterol enrichment did not alter the membrane or cytosolic content of PKC-α under basal conditions (Figure 7). PMA did not increase the membrane content of PKC-α but did increase a higher molecular weight PKC-α isoform (presumably a phosphorylated form of PKC-α), which was also unchanged by increasing the cholesterol content of the cell. However, the effects of cholesterol enrichment on PKC-δ were unique. Cholesterol enrichment reduced membrane content of PKC-δ under basal conditions. PMA induced translocation of PKC-δ from cytosol to membrane; this effect was augmented in cholesterol-enriched cells. Cholesterol enrichment did not significantly alter the cytosolic content of PKC-δ under basal or stimulated conditions (Figure 7). Smooth muscle cells possess little PKC-ζ, which remained unchanged with respect to cholesterol enrichment or stimulation by PMA (data not shown). Thus, these data indicate that (1) smooth muscle cells contain principally PKC-α and PKC-δ isoforms, (2) PKC-δ is preferentially translocated by PMA, and (3) cholesterol

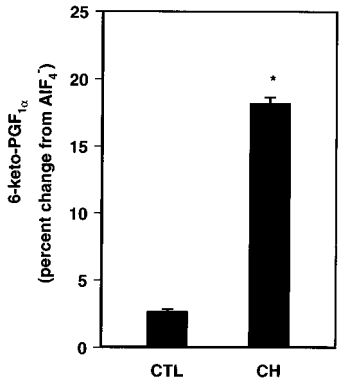

FIGURE 6: Enhancement of G-protein-mediated eicosanoid synthesis in cholesterol-enriched cells by mevalonate. Cells grown to confluent density in 35-mm wells were exposed to media containing cLDL and mevalonate (100 μM). Mevalonate was added to cells every 24 h. On day 7, cells were exposed to media alone or media containing AlF_4^- (30 mM). Supernatants were assayed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay. Data are expressed as percent change from control cells exposed to AlF_4^- (mean ± SEM, * = $p < 0.05$, CTL vs. cholesterol-enriched cells). This is representative of two experiments, each performed in triplicate.

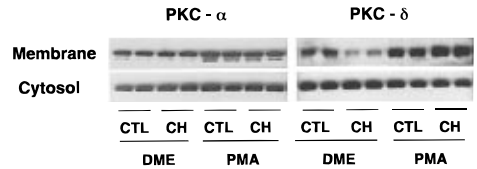

FIGURE 7: Enhancement of specific translocation of PKC-δ by cholesterol enrichment. Normal and cholesterol-enriched cells were exposed to media alone or media containing PMA (10^{-7} M). After 15 min, cells were harvested and subjected to subcellular fractionation. Membrane and cytosolic components were then analyzed for the relative mass of PKC-α and PKC-δ by immunoblotting. Each treatment was performed in duplicate.

enrichment enhances translocation of PKC-δ in response to PMA. These data support the concept that enhanced G-protein-mediated eicosanoid production may be due to enhanced PKC-δ activity.

Cholesterol Enrichment Does Not Alter Other Phospholipase A_2s. We next tested the alternative hypothesis that cholesterol enrichment augmented G-protein-mediated eicosanoid synthesis by enhancing the expression of other phospholipase A_2s found in smooth muscle cells. Smooth muscle cells possess 14.3.3ζ, which is a 30 kD protein possessing MAP-kinase-independent phospholipase A_2 activity. 14.3.3ζ has been cloned but has no known specific inhibitor (Zupan et al., 1992). As determined by Western analysis, cholesterol enrichment did not alter the expression of 14.3.3ζ (data not shown). Smooth muscle cells also possess a 40 kD MAP-kinase insensitive phospholipase A_2 that has not been cloned but whose activity is specifically inhibited by haloenol lactone suicide substrate (HELSS) (Lehman et al., 1993). However, the enhancement of PGI_2 synthesis by cholesterol-enriched cells exposed to AlF_4^- was not reduced by HELSS (data not shown). Collectively, these data exclude the induction of other phospholipase A_2s as a potential mechanism by which cholesterol enrichment enhances G-protein mediated phospholipase A_2 activity.

DISCUSSION

In arterial smooth muscle cells, heterotrimeric G-proteins couple extracellular vasoactive molecules, such as bradykinin, angiotensin II, ATP, and endothelin, to effectors that promote the release of intracellular second messengers, including nitric oxide, PGI_2, and cyclic AMP. The latter two second messengers are important since they mediate hydrolysis of intracellular CE and inhibit smooth muscle cell proliferation (Pomerantz & Hajjar, 1989a). In addition, they are involved in the maintenance of an "antiatherogenic" phenotype. Evidence to support this hypothesis is suggested by observations that cellular cholesterol accumulation impairs pertussis toxin-sensitive pathways leading to endothelium-dependent vasodilation (nitric oxide synthesis) (Shimokawa et al., 1991). The mechanisms by which cholesterol enrichment of cells alter G-protein-mediated eicosanoid production has been largely unexplored.

Herein, we show that smooth muscle cell eicosanoid synthesis occurs in response to ATP, AlF_4^-, and GTPγS (Figure 1) in a pertussis toxin-sensitive manner (Figure 2) by activating phospholipase A_2 but not phospholipase C (Figures 3 and 5). Gαi-2 (Winitz et al., 1994), Gαi-3 (Cantiello et al., 1990), and the $\beta\gamma$ heterodimer (Jelsema & Axelrod, 1987) stimulate phospholipase A_2 activity, supporting the premise that these G-proteins may mediate eicosanoid synthesis in vascular smooth muscle cells. These data parallel previous studies demonstrating that G-protein-mediated eicosanoid synthesis occurs by pertussis toxin-sensitive G-proteins coupled to phospholipase A_2 in endothelial cells (Garcia et al., 1992; Gerritsen & Mannix, 1990), macrophages (Balsinde et al., 1994; DuBourdieu & Morgan, 1990), and fibroblasts (Burch & Axelrod, 1987). The biphasic effects of G-protein agonists on smooth muscle cell eicosanoid production (Figure 1 and Rosenstock et al. (Rosenstock et al. 1996) and Gerritsen and Mannix (Gerritsen & Mannix, 1990)) suggests that more than one G-protein mediates eicosanoid production. Data to support this hypothesis are the observations that the stimulatory effect of low agonist concentrations of ATP are inhibited by pertussis toxin (Figure 2 and Rosenstock et al. (Rosenstock et al., 1996)), while the inhibitory effects of high agonist concentrations on eicosanoid production (Figure 1 and Rosenstock et al. (Rosenstock et al., 1996) and Gerritsen & Mannix (Gerritsen and Mannix, 1990)) is pertussis toxin-insensitive (Trent et al., 1996). Since it is doubtful that high agonist concentrations (>100 mM) are achieved, G-protein-linked eicosanoid synthesis in smooth muscle cells most likely occurs predominantly by a pertussis toxin-sensitive G-protein linked to phospholipase A_2 and not phospholipase C. It is noteworthy that G-protein receptor ligands are also capable of stimulating PLC in endothelial cells (Goligorsky et al., 1989; Garcia et al., 1993; Pollock et al., 1988), fibroblasts (L'Allemain et al., 1986), and smooth muscle cells (Schelling et al., 1994; Schneck et al., 1994).

In previous studies, we demonstrated that cholesterol enrichment reduces PGI_2 generation by inhibiting phospholipase A_2 (Pomerantz & Hajjar, 1989b), competitive inhibition of arachidonic acid release by enrichment of cell membrane phospholipids in linoleic acid (Pomerantz & Hajjar, 1989b), reducing the expression of PGHS-1 (Pomerantz et al., 1993), and reducing transcriptional activation of PGHS-2 in response to PDGF (Pomerantz et al., 1993). The latter effect may be due to the reduced ability of PDGF to stimulate MAP kinase activity (Pomerantz et al., 1997), a predominant mechanism by which PGHS-2 is activated (Hwang et al., 1996). In the studies reported herein, we

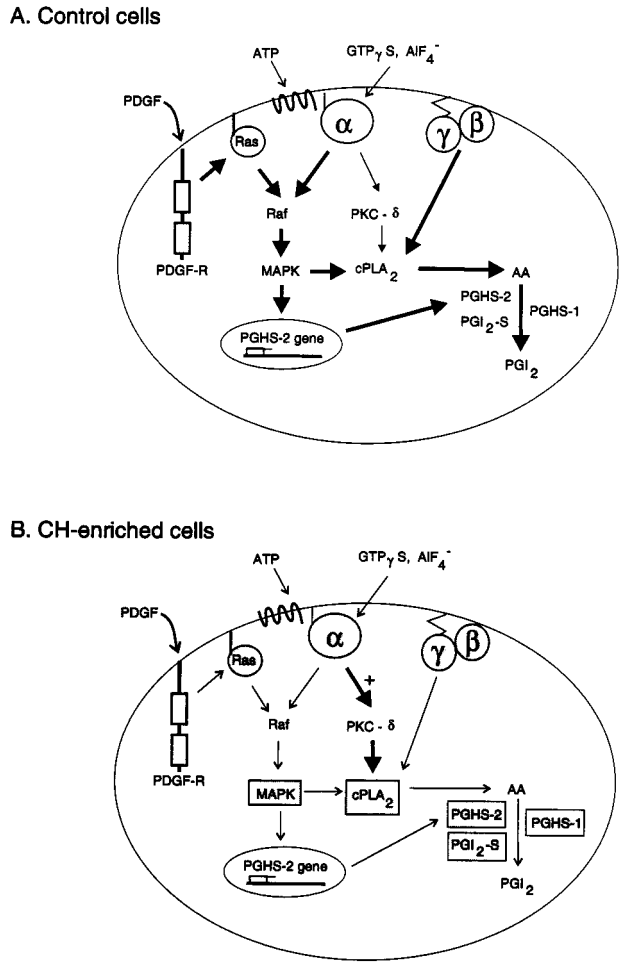

FIGURE 8: Alteration of G-protein-expression and signaling leading to PGI_2 production by cholesterol enrichment. (A) Eicosanoid production in control smooth muscle cells. Eicosanoid production can be enhanced by ligands binding to receptor tyrosine kinases (i.e., PDGF-R). Downstream signals via MAP-kinase enhance phospholipase A_2 activity, which increases substrate availability to PGHS-1 synthase. PDGF also enhances transcription of PGHS-2, further enhancing eicosanoid output. G-protein α-subunits stimulate phospholipase A_2 indirectly by stimulating raf (Johnson et al., 1994). G-$\beta\gamma$ heterodimers may stimulate phospholipase A_2 directly, or indirectly through p21ras. Phospholipase A_2 is also be activated by PKC-α, PKC-δ, or PKC-ζ. Under normal conditions, MAP-kinase-dependent processes leading to PGI_2 synthesis predominate (shown as bold) with PKC-dependent mechanisms having a smaller contribution toward the total eicosanoid output. (B) Eicosanoid production in cholesterol-enriched smooth muscle cells. Shaded figures are those proteins whose masses or activities are reduced following cholesterol enrichment. Cholesterol enrichment reduces G-protein-mediated or receptor tyrosine kinase-mediated MAP-kinase activation, $cPLA_2$ activation, induction of PGHS-2 gene expression, and PGHS-1 expression. However, cholesterol enrichment may augment G-protein-mediated eicosanoid metabolism by enhancing PKC-δ activity (shown as bold). Therefore, total eicosanoid output will be dependent upon the contribution of specific pathways leading to phospholipase A_2 activation.

extend these studies and demonstrate that cholesterol enrichment augments G-protein-mediated eicosanoid generation. This occurs in spite of the fact that cholesterol enrichment reduces heterotrimeric G-protein and p21ras content in smooth muscle cells and reduces the ability of AlF_4^- and PDGF to augment MAP kinase activity (Pomerantz et al., 1997). These findings suggest that cholesterol enrichment may augment other pathways linked to phospholipase A_2 or that it induces the synthesis of other phospholipases A_2. Since phospholipase A_2 activity and PGI_2 production is enhanced in cholesterol enriched cells exposed to pertussis toxin, cholesterol enrichment may upregulate a pertussis toxin-*insensitive* phospholipase A_2-activating pathway. Additional experiments support this mechanism. First, treatment of cholesterol-enriched cells with mevalonic acid (the product of HMG-CoA reductase) potentiated the effect of cholesterol enrichment on AlF_4^--induced PGI_2 synthesis (Figure 6). Since mevalonate also prevented cholesterol enrichment from reducing the membrane content of Gαi (Pomerantz et al., 1997), we interpret this finding to suggest that, in addition to reducing G-protein expression, cholesterol enrichment induces a pathway that enhances phospholipase A_2 activity independently of MAP kinase.

Two lines of evidence suggest that enhanced eicosanoid synthesis in response to G-protein agonists in cholesterol-enriched cells is due to enhanced protein kinase C. First, augmented PGI_2 production in response to AlF_4^- in cholesterol-enriched cells relative to controls was inhibited by calphostin C. Secondly, Western analysis revealed that cholesterol enrichment specifically reduced the basal membrane content of PKC-δ but enhanced the translocation of PKC-δ, but not other PKC isoforms, from the cytosol to the plasma membrane in response to PMA. These data support the premise that cholesterol enrichment enhances G-protein-mediated phospholipase A_2 activity by enhancing PKC-δ activity.

It has been well established that PKC-α, PKC-δ, or PKC-ζ stimulate phospholipase A_2 activity (Visnjic et al., 1995; Church et al., 1993) and eicosanoid production (Davies et al., 1984; Church et al., 1993). Herein, we demonstrate that this process is augmented following cholesterol enrichment. However, the mechanisms by which cholesterol enrichment enhances PKC-δ activity are currently under investigation. *In vivo* experiments demonstrate that cholesterol feeding of rabbits increases PKC activity in smooth muscle cells even in the presence of vitamin E (Sirikci et al., 1996), excluding oxysterols as a mechanism for enhanced PKC activity. The presence of oxidized lipid is not a mechanism for enhanced PKC activity in these studies since neither LDL nor cholesterol-enriched cells contain detectable levels of cholesterol oxides (Pomerantz et al., 1997). Alterations in the transcription of PKC-δ mRNA are also unlikely since the cytosolic content of PKC-δ was unchanged, while the membrane component of PKC-δ was reduced. In addition, since PKC-δ is not calcium-dependent (Andrea & Walsh, 1992), it is unlikely that enhanced calcium flux as occurs in cholesterol-enriched cells (Bialecki et al., 1991) mediates enhanced PKC-δ activity. It is also unlikely that cholesterol-enriched cells generate elevated amounts of diacylglycerols, since G-protein agonists such as ATP and AlF_4^- do not significantly stimulate phospholipase C activity in smooth muscle cells, whether measured as accumulated inositol phosphates (Figure 3) or phosphorylated PLC-γ (Figure 5).

Finally, enhanced phospholipase A_2 activity in cholesterol-enriched cells was not due to induction of other smooth muscle cell phospholipases. These include 14.3.3ζ, a member of the 14.3.3 class of chaparones that possesses phospholipase A_2 activity (Zupan et al., 1992) or the p40 kD phospholipase A_2, which is specifically inhibited by HELSS (Lehman et al., 1993). Although p60src has been implicated in G-protein-mediated phospholipase activation

(Ishida et al., 1995), cholesterol enrichment does not alter membrane-associated p60src (Pomerantz et al., 1997).

Collectively, our data support the hypothesis that cholesterol enrichment enhances G-protein mediated eicosanoid synthesis by promoting the activation of PKC-δ and, hence, activation of phospholipase A_2. A model describing the cholesterol enrichment on cell signaling leading to eicosanoid synthesis is depicted in Figure 8. Our findings suggest that the net eicosanoid output by cholesterol- enriched cells can be a result of inhibitory (e.g., PDGF) as well as stimulatory (e.g., ATP) signals. These results may explain the divergent effects of arterial cholesterol accumulation on vascular eicosanoid metabolism.

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