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Lysophosphatidic Acid Regulation of Cyclic AMP Accumulation in Cultured Human Airway Smooth Muscle Cells

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SUMMARY

The effects of the simple bioactive lipid mediator lysophosphatidic acid (LPA) on cAMP accumulation were investigated in cultured human airway smooth muscle cells (ASMC). Pretreatment of cells with LPA induced an increase in subsequent stimulation of cAMP accumulation by forskolin and by isoproterenol. When included during the assay of cAMP accumulation rather than as a pretreatment, LPA inhibited forskolin stimulation but enhanced isoproterenol stimulation. Both effects of LPA on forskolin stimulation were completely blocked by pertussis toxin treatment, whereas the effects on isoproterenol stimulation appeared relatively insensitive to pertussis toxin. The protein kinase C activator phorbol-12-myristate-13-acetate (PMA) sensitized forskolin stimulation to a similar extent as did LPA, and the combination of LPA plus PMA caused markedly more sensitization than either agent alone. In contrast, PMA inhibited isoproterenol stimulation and markedly decreased the sensitization induced by LPA. Serum also induced sensitization, and sensitization by LPA plus serum was no greater than that with LPA alone. LPA-induced sensitization appeared to be independent of protein kinase C activation because it was unchanged in cells treated to down-regulate protein kinase C. LPA also stimulated polyphosphoinositide hydrolysis, and this stimulation was partially inhibited by pertussis toxin treatment. These results suggest that LPA activates receptors coupled to both the pertussis toxin-sensitive G protein G_i and the pertussis toxin-insensitive G protein G_q. The complex effects of LPA, PMA, and pertussis toxin on cAMP accumulation in these cells are consistent with the expression of the type 2 isozyme of adenylyl cyclase in these cells.

cAMP is well established as an important second messenger regulating a wide variety of cellular functions, including energy metabolism, muscle contraction, neuronal signaling, and cell growth. Modulation of cAMP levels is of particular therapeutic significance in the regulation of airway smooth muscle contractility (1, 2). β-Adrenergic receptor agonists increase cAMP by stimulating the adenylyl cyclase enzymes responsible for its synthesis. Methylxanthines increase cAMP by inhibiting the phosphodiesterase enzymes that degrade cAMP. Both types of agents relax airway smooth muscle and induce clinically important bronchodilation (1, 2) Information on additional agents and mechanisms involved in regulation of cAMP metabolism in airway smooth muscle has the potential to identify new targets for therapeutic intervention in pulmonary diseases.

Recent studies have characterized an adaptive change in cAMP regulation referred to as "sensitization" (3–8), the phenomenon in which pretreatment of cells with agents that do not stimulate adenylyl cyclase leads to an increase in subsequent cAMP accumulation in response to other agents that do stimulate adenylyl cyclase. For example, we have shown sensitization to occur after activation of receptors that couple to inhibition of adenylyl cyclase, such as α_2-adrenergic receptors (3); after activation of receptors coupled to polyphosphoinositide hydrolysis, such as M_3 muscarinic and H_1 histamine receptors (4, 5); and after direct activation of protein kinase C with phorbol esters and related compounds (5). We also found that a factor present in serum could induce sensitization in two glial cell lines (6, 7), and we recently presented evidence indicating that the serum factor is LPA or a closely related compound present in serum bound to albumin (8).

LPA can exert a wide range of physiological effects on various mammalian cells, including mitogenesis, cell shape changes, platelet activation, and smooth muscle contraction (9, 10). LPA is released from activated platelets, and numerous studies have shown it to be an important active component of serum (7, 11–14). LPA can activate multiple second-messenger systems in cells, including inhibition of adenylyl cyclase, stimulation of polyphosphoinositide hydrolysis, elevation of intracellular Ca^{2+}, release of arachidonic acid, and

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ABBREVIATIONS: LPA, lysophosphatidic acid; PMA, phorbol-12-myristate-13-acetate; ASMC, airway smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

activation of *ras* and mitogen-activated protein kinases (9, 10). Although the receptor for LPA has not been isolated, the pertussis toxin sensitivity of many of its cellular effects suggests that it activates a member of the G protein-coupled receptor family, and a putative receptor protein has been identified by photoaffinity labeling (15).

In a previous study (8), we screened a variety of cell types for the ability of LPA to induce sensitization of cAMP accumulation. We found that LPA induced sensitization in cultured human ASMC, although this response was not further characterized. Because of the possible physiological significance of LPA in regulating airway cell function, we further investigated its effects in these cells. The results of the present study characterize in greater detail the effects of LPA on cAMP accumulation in human ASMC.

Experimental Procedures

Materials. [^{3}H]Adenine was obtained from NEN (Wilmington, DE); [^{3}H]inositol from Amersham (Arlington Heights, IL); forskolin from Calbiochem (La Jolla, CA); pertussis toxin from List Biological Laboratories (Campbell, CA); LPA, isoproterenol, PMA, and other biochemicals from Sigma Chemical Co. (St. Louis, MO); and fetal bovine serum and growth media from GIBCO-BRL (Grand Island, NY).

Cell culture. Human ASMC (tracheal) isolated as previously described (16) were kindly provided by Dr. Michael Kotlikoff (University of Pennsylvania). The cells were cultured to confluence in DMEM containing 10% fetal bovine serum. Cells passaged 2–12 times were used for the experiments.

cAMP accumulation assays. The [^{3}H]adenine prelabeling procedure of Shimizu *et al.* (17) was used to measure cAMP accumulation by intact cells, essentially as described in our previous studies of sensitization (6–8). Cells were grown to confluence on 35-mm dishes. In some experiments, cells were pretreated for 24 hr in the absence or presence of 100 ng/ml pertussis toxin. Cells were washed and then incubated for 1 hr in DMEM buffered to pH 7.4 with 20 mM HEPES containing 2 μCi [^{3}H]adenine in the absence or presence of various concentrations of LPA, PMA, or Serum. Cells were then washed with DMEM-HEPES and incubated for 2 min at 37° in DMEM-HEPES containing 0.1 mM 3-isobutyl-1-methylxanthine, in the absence or presence of 10 μM isoproterenol or 30 μM forskolin to stimulate cAMP formation. In some experiments, LPA was included during this stimulation step rather than during labeling with [^{3}H]adenine. The stimulation medium was aspirated, and cellular nucleotides were extracted with 5% trichloroacetic acid. The trichloroacetic acid extracts were subjected to sequential chromatography over Dowex and alumina columns to separate [^{3}H]cAMP from [^{3}H]ATP. Radioactivity in the samples was quantified by liquid scintillation counting. cAMP accumulation was calculated as percent conversion of [^{3}H]ATP to [^{3}H]cAMP. This assay does not give absolute values for cAMP accumulation but has the advantage of intrinsically normalizing the data for changes in cell number or viability. In the figures, percent conversion values for cAMP accumulation in treated cells have been normalized to the values in control cells, and the data are expressed as fold of control. Typical percent conversion values in control cells were as follows: basal, 0.1%; 30 μM forskolin, 0.4%; and 10 μM isoproterenol, 1.0%.

Polyphosphoinositide hydrolysis assays. [^{3}H]Inositol phosphate formation assays were adapted from the procedure of Nakahata *et al.* (18). Cells grown to confluence on 35-mm plastic culture dishes were incubated overnight with [^{3}H]inositol (2 μCi/dish) in growth medium. Cells were washed and then incubated for 60 min in 1 ml DMEM-HEPES containing 10 mM LiCl in the absence or presence of LPA for 20 min. The cells were washed one time, and the reaction was stopped by the addition of 500 μl methanol. The cells were scraped from the dish, placed in glass tubes, and extracted with chloroform, water, and additional methanol (final ratio of reagents, 1:0.9:1, v/v/v). The phases were separated by centrifugation, and 1.4 ml of the aqueous phase was transferred to Dowex 1X8 columns. Separation of aqueous [^{3}H]inositol-labeled compounds was performed by sequential elution with ammonium formate and formic acid. Total [^{3}H]inositol phosphates were collected as a single fraction, and radioactivity was determined by liquid scintillation counting. Values for [^{3}H]inositol phosphates formation are presented as percent conversion of [^{3}H]inositol phospholipids to [^{3}H]inositol phosphates.

Data analysis. Data in all figures are the mean ± standard error from at least three experiments, each performed in duplicate or triplicate. Where error bars are not apparent, the value is smaller than the symbol. Analysis of concentration-response curves was made by computerized nonlinear regression analysis with GraphPad InPlot (San Diego, CA).

Results

Characterization of sensitization induced by pretreatment with LPA. In a previous study, we demonstrated that pretreatment of cultured human ASMC with 1 μM LPA or 5% fetal bovine serum induced a 2.5–3-fold sensitization of subsequent stimulation of cAMP accumulation by the direct adenylyl cyclase activator forskolin (8). The concentration dependence of LPA sensitization of stimulation by forskolin and by the β-adrenergic receptor agonist isoproterenol was investigated (Fig. 1). Pretreatment of human ASMC with LPA for 60 min induced a concentration-dependent sensitization of both forskolin (30 μM) and isoproterenol (10 μM) stimulation. For forskolin stimulation, half-maximal sensitization occurred with 500 nM LPA, and the maximal effect with 100 μM LPA was approximately 2.5-fold. For isoproterenol stimulation, half-maximal sensitization was observed with 30 nM LPA, and the maximal effect with 100 μM LPA was approximately 1.8-fold sensitization.

In the two glial cell lines studied previously, sensitization of both forskolin and isoproterenol stimulation by LPA and by serum was essentially completely blocked in cells pretreated with pertussis toxin (6–8, 19). The pertussis toxin sensitivity of LPA-induced sensitization in human ASMC was assessed (Fig. 2). Overnight pretreatment of cells with 100 ng/ml pertussis toxin to inactivate pertussis toxin-sensitive G proteins prevented the sensitization of forskolin stimulation induced by all concentrations of LPA. For isoproterenol stimulation, the nearly maximal sensitization induced by 100 nM LPA was inhibited markedly, but not completely, by pertussis toxin. In contrast, the sensitization induced by 100 μM LPA was not inhibited by pretreatment with pertussis toxin.

Characterization of LPA effects when present during assay of cAMP accumulation rather than as a pretreatment. The effects of LPA on cAMP accumulation were assessed when included in the stimulation assay rather than as a pretreatment (Fig. 3). LPA alone had little effect on the low level of basal cAMP accumulation (data not shown). Forskolin (30 μM) stimulated cAMP accumulation 3.5-fold over basal in the absence of LPA, and LPA inhibited the stimulation by forskolin. Half-maximal inhibition was observed with approximately 10 nM LPA, and maximal inhibition of 32% was observed with 100 μM LPA. Isoproterenol (10 μM) stimulated cAMP accumulation by 7-fold, and LPA en-

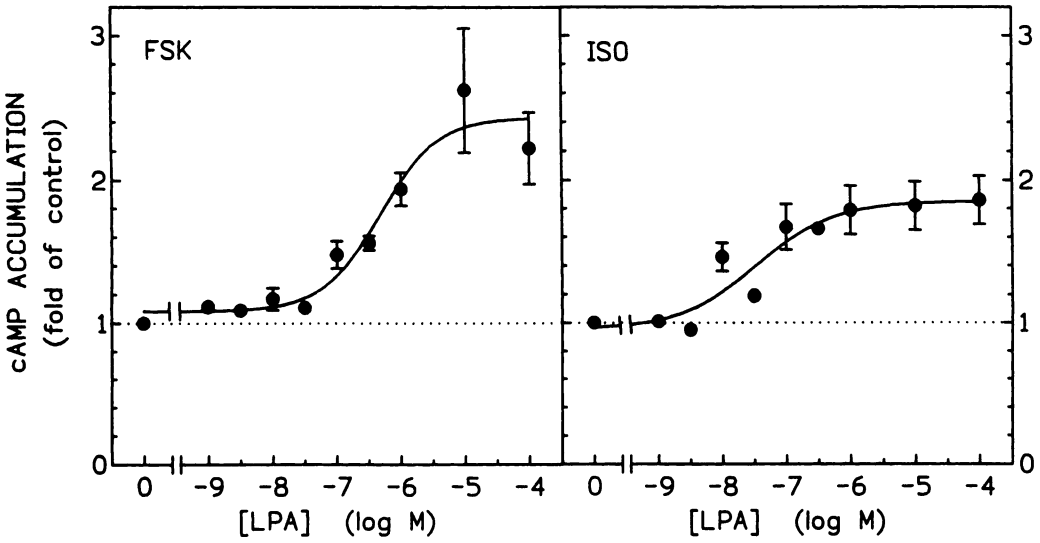

Fig. 1. LPA concentration dependence of sensitization of forskolin and isoproterenol stimulation of cAMP accumulation. Cells were incubated for 60 min in the absence (0) or presence of the indicated concentrations of LPA and then washed. cAMP accumulation stimulated by 30 μM forskolin (*FSK*) or 10 μM isoproterenol (*ISO*) was measured in 2-min assays.

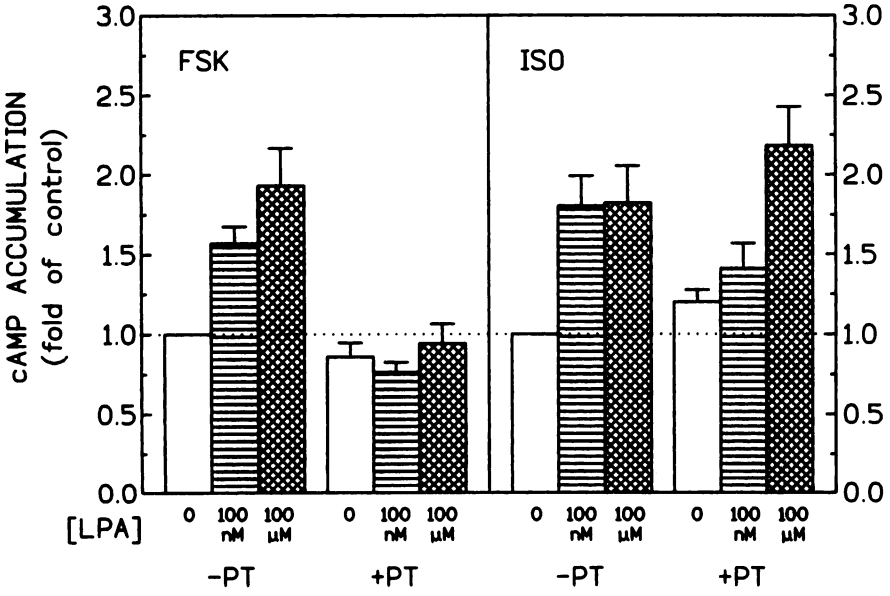

Fig. 2. Pertussis toxin sensitivity of LPA-induced sensitization. Cells were incubated overnight in the absence (−*PT*) or presence (+*PT*) of 100 ng/ml pertussis toxin and then for 60 min in the absence (0) or presence of 100 nM or 100 μM LPA. Cells were washed, and cAMP accumulation stimulated by 30 μM forskolin (*FSK*) or 10 μM isoproterenol (*ISO*) was measured in 2-min assays.

hanced the stimulation by isoproterenol. Half-maximal enhancement was observed with approximately 10 nM LPA, and maximal enhancement of 2.2-fold was observed with 100 μM LPA.

The pertussis toxin sensitivity of the effects of LPA when included during the assays of forskolin and isoproterenol stimulation was investigated (Fig. 4). Overnight pretreatment with 100 ng/ml pertussis toxin slightly increased forskolin stimulation in the absence of LPA, and the inhibition of forskolin stimulation by LPA was completely eliminated. Pertussis toxin pretreatment also increased stimulation by isoproterenol in the absence of LPA; however, the enhancement of isoproterenol stimulation by LPA was only slightly reduced by pertussis toxin.

Effects of protein kinase C activation on cAMP accumulation and on LPA-induced sensitization. In our previous studies, direct activation of protein kinase C by PMA induced sensitization of cAMP accumulation in some cells (5, 6) but not in others (3, 7). The effects of PMA on stimulation of cAMP accumulation by forskolin and isoproterenol and on sensitization of that stimulation by LPA in human ASMC were investigated (Fig. 5). Pretreatment of these cells for 60 min with PMA alone induced a concentration-dependent sensitization of forskolin stimulation (Fig. 5, *top*). Half-maximal sensitization occurred with 2 nM PMA, and maximal sensitization with 1 μM PMA was 3.0-fold. Pretreatment of cells with 1 μM LPA alone induced 3.7-fold sensitization in these experiments, which was somewhat more than that in Fig. 1. Pretreatment with increasing concentrations of PMA in the presence of 1 μM LPA induced a marked sensitization of forskolin stimulation. Half-maximal enhancement of LPA-induced sensitization by PMA was observed with 2 nM PMA, and combined pretreatment with 1 μM PMA and 1 μM LPA led to a striking 10.8-fold sensitization of forskolin stimulation.

The effects of PMA pretreatment on isoproterenol stimulation were quite different. Pretreatment for 60 min with PMA alone induced a modest (10–15%) inhibition of isoproterenol stimulation (Fig. 5, bottom panel). Pretreatment of cells with 1 μM LPA alone induced 1.5-fold sensitization in these experiments, somewhat smaller than that in Fig. 1 above. Pretreatment with increasing concentrations of PMA

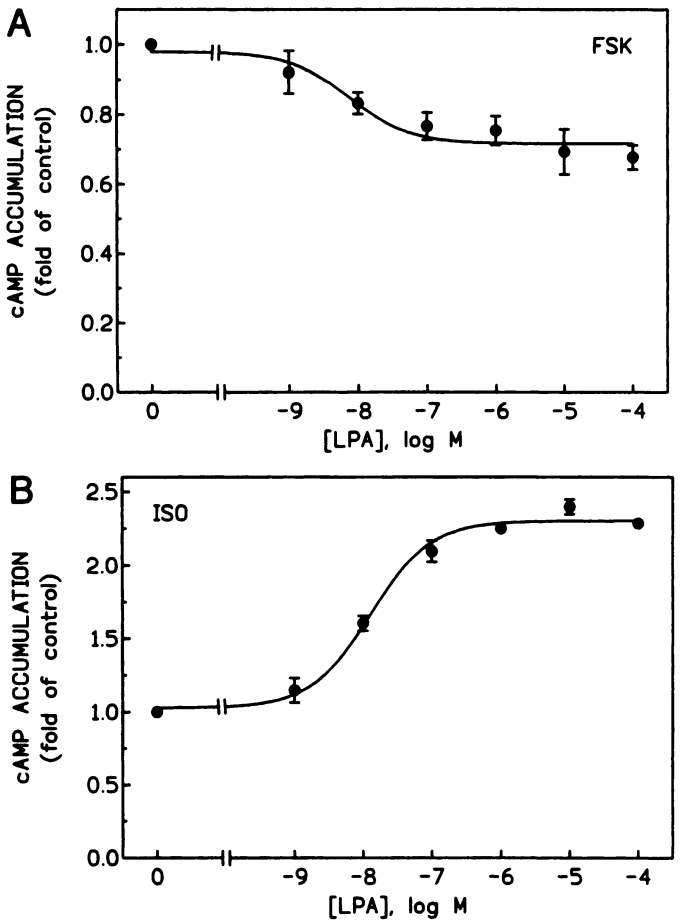

Fig. 3. Acute effects of LPA when included during the assay of cAMP accumulation. Stimulation of cAMP accumulation by 30 μM forskolin (*FSK*; *top*) and by 10 μM isoproterenol (*ISO*; *bottom*) was measured in 2-min assays in the absence (0) or presence of the indicated concentrations of LPA.

in the presence of 1 μM LPA reduced the sensitization induced by LPA. Half-maximal inhibition of the LPA-induced sensitization by PMA was observed with 3 nM PMA, and sensitization by 1 μM LPA in the presence of 1 μM PMA was only 1.1-fold.

Comparison of sensitization induced by LPA, PMA, and serum. We initially described the ability of serum to induce sensitization of cAMP accumulation in two glial cell lines (6, 7); not until later was the serum sensitization factor for these cells identified as LPA (8). The ability of serum to induce sensitization in human ASMC was tested and compared with the effects of LPA and PMA, alone and in combinations (Fig. 6). Fetal bovine serum (5%) induced sensitization of cAMP accumulation in human ASMC, with 2.4-fold sensitization of forskolin stimulation and 1.7-fold sensitization of isoproterenol stimulation. LPA (1 μM) was somewhat more effective than serum in the case of forskolin stimulation (3.7-fold) and slightly less effective than serum in the case of isoproterenol stimulation (1.5-fold). Sensitization with the combination of serum plus LPA was no more than that with LPA alone, consistent with LPA being the relevant sensitization factor in serum for human ASMC. In contrast to the lack of additivity for the combination of serum and LPA, the combination of PMA with either serum or LPA led to additive or more-than-additive effects on forskolin stimulation. These results suggest that PMA induces sensitization via a different pathway than that induced by serum and LPA. In the case of isoproterenol stimulation, the combination of PMA with either serum or LPA eliminated the sensitization induced by these agents alone.

Effects of protein kinase C down-regulation on LPA- and PMA-induced changes in cAMP accumulation. We assessed the effects of overnight pretreatment of human ASMC with 10 μM PMA to induce protein kinase C down-regulation on the subsequent ability of PMA and LPA to induce changes in cAMP accumulation stimulated by forskolin (Fig. 7, *top*) and by isoproterenol (Fig. 7, *bottom*). In the case of forskolin stimulation in control cells with intact protein kinase C, 1 μM PMA alone induced 3.2-fold sensitization, 1 μM LPA alone induced 4.2-fold sensitization, and the combination of LPA and PMA induced 17.0-fold sensitization. In protein kinase C down-regulated cells, forskolin stimulation in the absence of additional PMA or LPA pretreatment was increased 2.2-fold. PMA did not induce further sensitization, consistent with down-regulation of protein kinase C. However, LPA retained its full ability to induce sensitization, causing a 6.3-fold increase over the activity in cells not exposed to LPA. The combination of LPA plus PMA did not induce any greater effect than LPA alone, consistent with the expected down-regulation of protein kinase C.

In the case of isoproterenol stimulation in control cells with protein kinase C intact, PMA induced a 42% decrease, a significantly greater decrease than that observed in the experiments in Fig. 3. LPA induced 1.6-fold sensitization, and this LPA-induced sensitization was reduced to 1.3-fold in the presence of PMA. In protein kinase C down-regulated cells, isoproterenol stimulation in the absence of additional PMA or LPA pretreatment was decreased by 44%. PMA induced little further desensitization (51%), consistent with down-regulation of protein kinase C. However, LPA was still able to induce sensitization in these cells, causing a somewhat greater sensitization (2.1-fold) than that in control cells (1.6-fold). Inclusion of PMA during the LPA pretreatment had little effect on LPA sensitization in protein kinase C down-regulated cells (2.3-fold sensitization), again consistent with protein kinase C down-regulation. The effects of protein kinase C down-regulation on serum-induced sensitization were essentially identical to those on LPA-induced sensitization (data not shown).

Effects of LPA on polyphosphoinositide hydrolysis. The ability of LPA to stimulate polyphosphoinositide hydrolysis in human ASMC was tested (Fig. 8). LPA stimulated inositol phosphate formation in a concentration-dependent manner. Half-maximal stimulation occurred with 0.4 μM LPA, and maximal stimulation to 4.2% conversion (5.1-fold stimulation over basal) was observed with 100 μM LPA. Pretreatment of cells with pertussis toxin inhibited but did not eliminate the stimulation by LPA. After treatment with pertussis toxin, half-maximal stimulation occurred with 1.1 μM LPA, and the maximal stimulation was reduced by 51% to 2.1% conversion (3.3-fold stimulation over basal).

Discussion

The results of the present study document multiple effects of the simple lipid mediator LPA on cAMP accumulation in human ASMC. It should be noted that these effects on cAMP

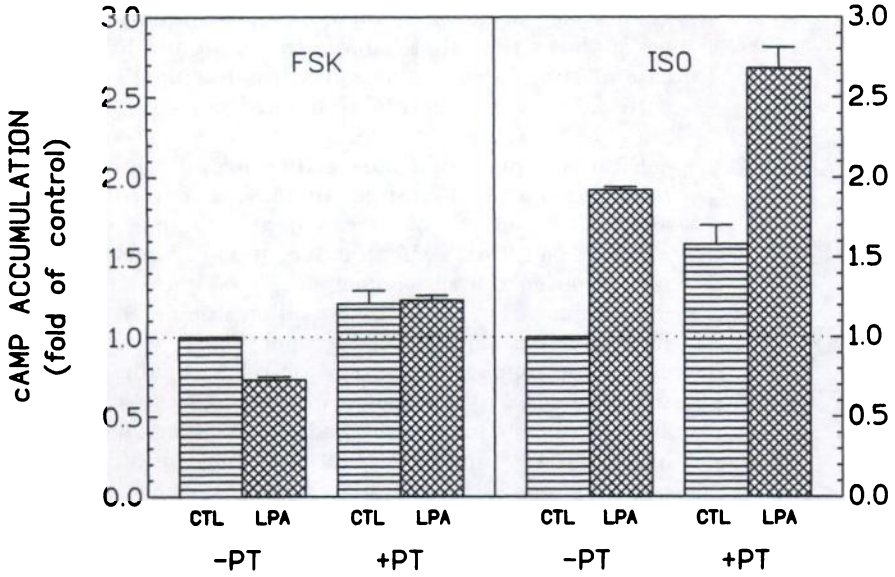

Fig. 4. Effects of pertussis toxin on LPA-induced inhibition of forskolin stimulation and enhancement of isoproterenol stimulation. Cells were incubated overnight in the absence (*−PT*) or presence of 100 ng/ml pertussis toxin (*+PT*). Stimulation of cAMP accumulation by 30 μM forskolin (*FSK*) and by 10 μM isoproterenol (*ISO*) was measured in the absence (*CTL*) or presence of 1 μM LPA in 2-min assays.

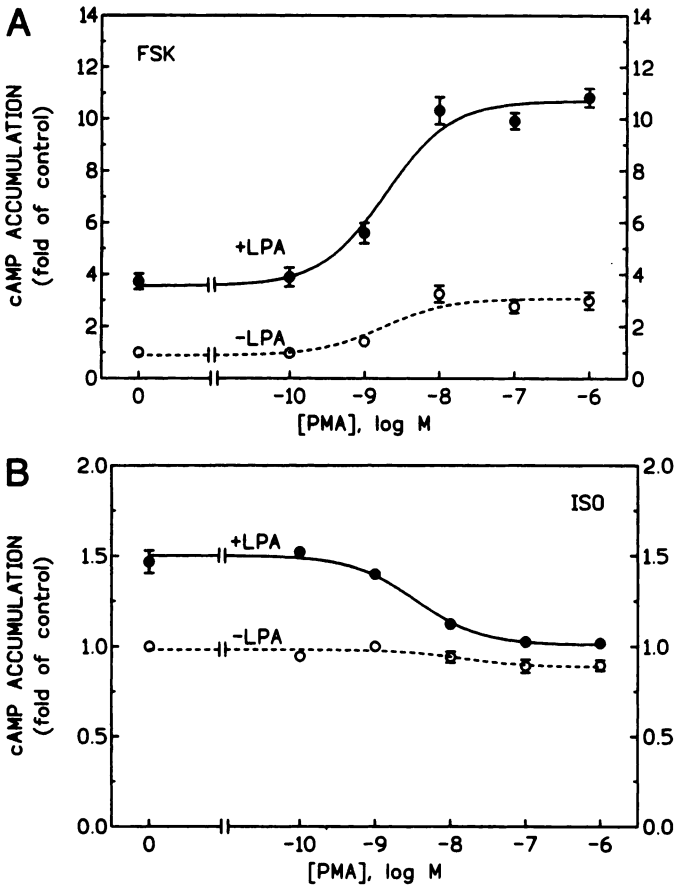

Fig. 5. Effects of PMA on forskolin- and isoproterenol-stimulated cAMP accumulation and on LPA-induced sensitization of that stimulation. Cells were pretreated for 60 min in the absence (*−LPA*) or presence (*+LPA*) of 1 μM LPA in the absence (0) or presence of the indicated concentrations of PMA, washed, and stimulation of cAMP accumulation by 30 μM forskolin (*FSK*) and by 10 μM isoproterenol (*ISO*) was measured in 2-min assays.

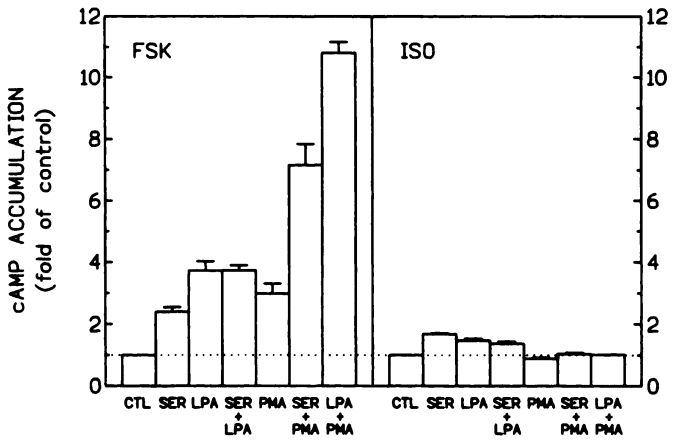

Fig. 6. Comparison of sensitization induced by serum, LPA, and PMA. Cells were pretreated for 60 min in the absence (*CTL*) or presence of 5% fetal bovine serum (*SER*), 1 μM LPA, or 1 μM PMA, alone or in combination. Cells were washed, and stimulation of cAMP accumulation by 30 μM forskolin (*FSK*) and by 10 μM isoproterenol (*ISO*) was measured in 2-min assays.

accumulation are most likely effects on cAMP synthesis by adenylyl cyclase rather than cAMP degradation by phosphodiesterases because all assays were conducted in the presence of a phosphodiesterase inhibitor. When included during the assays of cAMP accumulation, LPA inhibited forskolin stimulation but enhanced stimulation by isoproterenol. These two effects are what would be predicted if these cells express the type 2 isozyme of adenylyl cyclase and if LPA activates a receptor coupled to the inhibitory G protein G_i (Fig. 9). The type 2 isozyme is inhibited by the α subunit of G_i, but stimulation of the type 2 isozyme by the α subunit of G_s is potentiated by the $\beta\gamma$ subunits of various G proteins (20–24). In the case of forskolin stimulation, adenylyl cyclase is activated directly rather than through $G_s\alpha$; therefore, the inhibitory effect of the $G_i\alpha$ subunit predominates, and inhibition is observed. In the case of isoproterenol stimulation, activated $G_s\alpha$ is the stimulus for adenylyl cyclase activation; the potentiation of stimulation by the $\beta\gamma$ subunits of both G_i and G_s appears to override the inhibition by $G_i\alpha$, and an increase in isoproterenol stimulation is observed.

The effects of the protein kinase C activator PMA on cAMP accumulation are also consistent with the presence of the type 2 isozyme of adenylyl cyclase in human ASMC. The type 2 isozyme can be stimulated and/or sensitized by PMA (22–

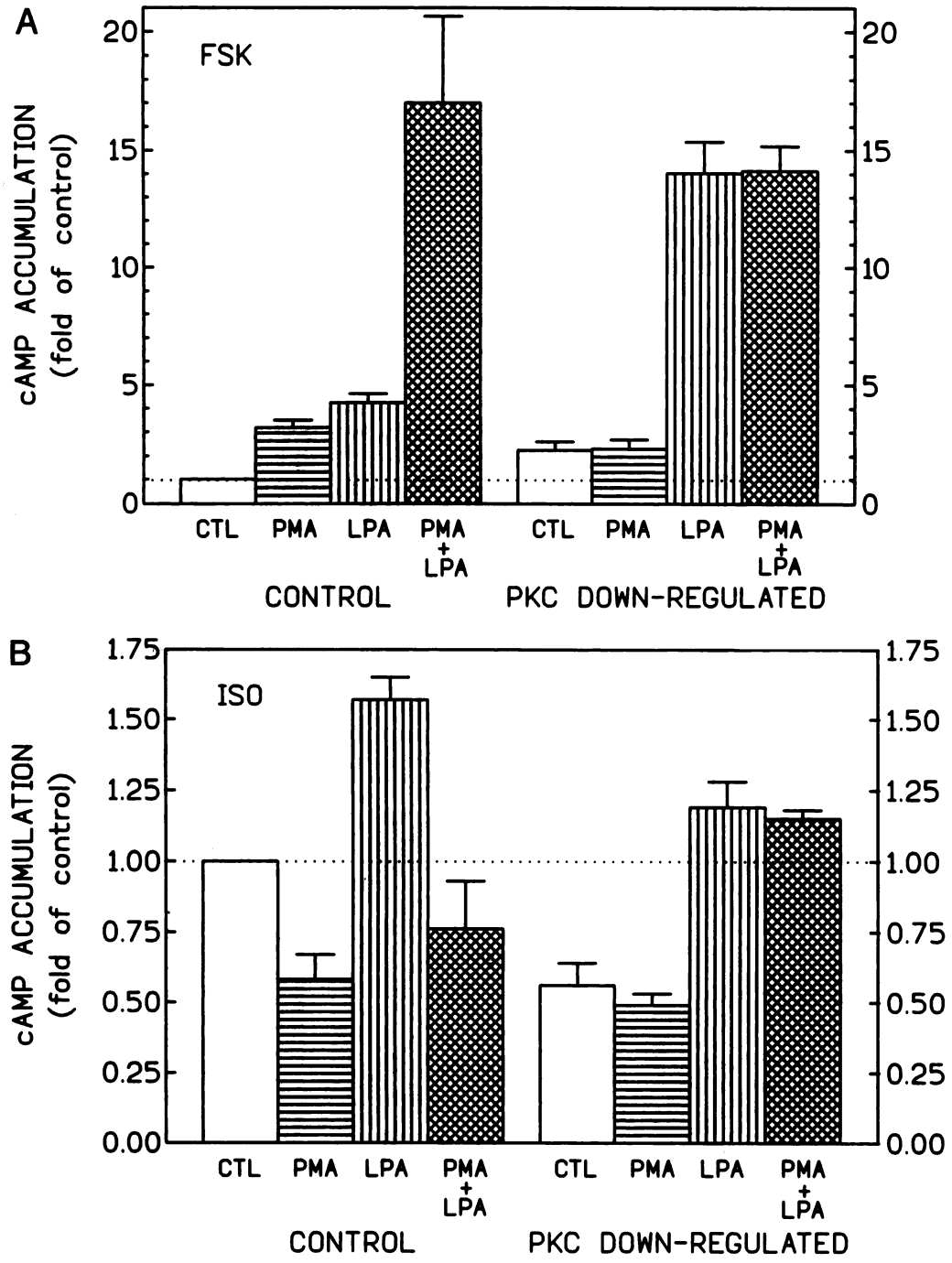

Fig. 7. Effects of protein kinase C down-regulation on PMA- and LPA-induced changes in cAMP accumulation. Cells were incubated overnight in the absence (*CONTROL*) or presence of 10 μM PMA to down-regulate protein kinase C (*PKC DOWN-REGULATED*). Cells were then pretreated for 60 min in the absence (*CTL*) or presence of 1 μM PMA, 1 μM LPA, or both PMA and LPA and washed, and stimulation of cAMP accumulation by 30 μM forskolin (*FSK*; *top*) and by 10 μM isoproterenol (*ISO*; *bottom*) was measured in 2-min assays.

26), and PMA-induced enhancement of forskolin stimulation was observed in these cells. However, in the case of isoproterenol, PMA decreased rather than enhanced the stimulation of cAMP accumulation. Protein kinase C activation has been shown to desensitize β-adrenergic receptor function in numerous systems (27, 28). We suggest that such PMA-induced desensitization at the level of the receptor may override the PMA-induced enhancement that occurs at the level of the adenylyl cyclase enzyme; however, this hypothesis remains to be tested.

Data from other studies support the presence of the type 2 adenylyl cyclase isozyme in ASMC. Northern blot analysis indicated the presence of mRNA for the type 2 isozyme in lung tissue (29). More recently, Pyne *et al.* (30) presented Western blot data indicating the presence of the type 2 isozyme in primary cultures of isolated guinea pig ASMC. Pian and Dobbs (31) recently reported the presence of type 2 adenylyl cyclase in rat lung alveolar cells and the ability of G_i protein-coupled receptor agonists to potentiate G_s protein-coupled receptor agonist stimulation of cAMP production, similar to what we propose for LPA.

Most but not all of the effects of LPA in other cells are inhibited by pertussis toxin (9, 10), indicating that the effects of LPA are mediated via activation of either G_i or G_o, the two major families of pertussis toxin-sensitive G proteins (32). The ability of pertussis toxin to prevent the LPA-induced inhibition of forskolin stimulation of cAMP accumulation is consistent with LPA receptor coupling to G_i. The pertussis toxin sensitiv-

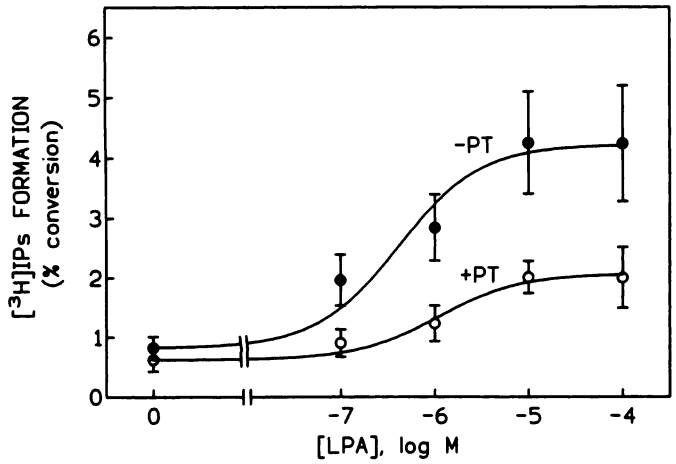

Fig. 8. LPA stimulation of polyphosphoinositide hydrolysis in the absence and presence of pertussis toxin pretreatment. Cells were incubated overnight with [^{3}H]inositol in the absence (*−PT*) or presence of 100 ng/ml pertussis toxin (*+PT*). Stimulation of [^{3}H]inositol phosphates ([^{3}H]IPs) formation was then determined in the absence (0) or presence of the indicated concentrations of LPA in 20-min assays. The average raw values for basal activity in control cells (−PT, 0) were 143 cpm/dish for the [^{3}H]IPs fraction and 22,300 cpm/dish for the [^{3}H]inositol phospholipid fraction.

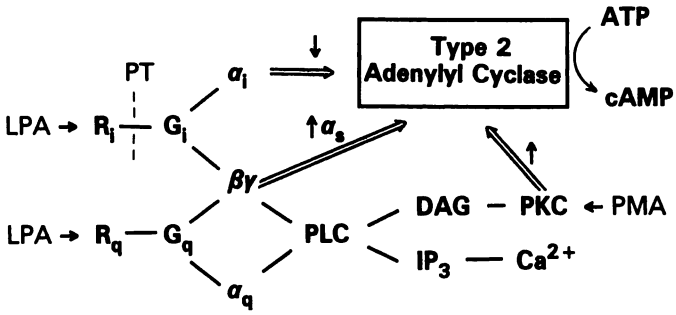

Fig. 9. Model for LPA regulation of cAMP accumulation in human ASMC. R_i represents an LPA receptor coupled to the inhibitory G protein G_i, and R_q represents an LPA receptor coupled to phospholipase C (*PLC*) by a pertussis toxin-insensitive G protein such as G_q; these could be different receptor subtypes or a single receptor capable of activating both G proteins. α_i, α_s, and α_q represent the α subunits of G_i, G_s, and G_q respectively. *Double arrows*, three proposed inputs to type 2 adenylyl cyclase regulation. *Up arrows*, stimulation or enhancement. *Down arrows*, inhibition. *PT*, pertussis toxin; *DAG*, diacylglycerol; *PKC*, protein kinase C; *IP3*, inositol trisphosphate.

ity of the sensitization of forskolin stimulation induced by pretreatment with LPA suggests that LPA-induced sensitization is also mediated by a pathway involving G_i.

The effects of pertussis toxin on LPA-induced modulation of isoproterenol stimulation of cAMP accumulation are more difficult to explain. Pertussis toxin alone increased subsequent stimulation by isoproterenol, presumably due to removal of a tonic level of inhibition mediated by G_i. However, pertussis toxin did not prevent the LPA-induced enhancement of isoproterenol stimulation as it did the LPA-induced inhibition of forskolin stimulation. If the cAMP accumulation in these cells is largely due to type 2 adenylyl cyclase, then the enhancement of isoproterenol stimulation by LPA is presumably due to $\beta\gamma$ subunits. The inability of pertussis toxin to block the LPA-induced enhancement suggests that the $\beta\gamma$ subunits responsible for the enhancement (at least in pertussis toxin-treated cells) are from a G protein other than G_i. An obvious possibility for the source of these $\beta\gamma$ subunits would be G_q, based on the ability of LPA to stimulate polyphosphoinositide hydrolysis by a pertussis toxin-independent pathway in these cells. In cells not treated with pertussis toxin, the inhibitory effect of the $G_i\alpha$ subunits may be approximately balanced by the enhancing effect of the co-released $G_i\beta\gamma$ subunits. Thus, removal of the effects of both the α and $\beta\gamma$ subunits of G_i by treatment with pertussis toxin might have little effect on the LPA enhancement of isoproterenol stimulation, leaving the $\beta\gamma$ subunits of G_q to enhance the stimulation by the activated $G_s\alpha$ subunit.

The ability of low concentrations of LPA to potentiate isoproterenol stimulation via a pertussis toxin-insensitive pathway when both are present during the assay (Figs. 3 and 4) is the most likely explanation for the pertussis toxin-insensitivity of the apparent sensitization of isoproterenol stimulation seen with 100 μM LPA (Fig. 2) Cells may not have washed adequately to completely remove LPA between the pretreatment and assay steps in these experiments. Any residual LPA that may have remained in the dish would then be able to potentiate isoproterenol stimulation during the subsequent assay, leading to an *apparent* sensitization that would be pertussis toxin-insensitive. The lower concentration of residual LPA after the pretreatment with 100 nM LPA would probably be insufficient to induce significant potentiation, and thus only the pertussis toxin-sensitive sensitization would be observed.

Fig. 9 is our working model for the effects of LPA when included during the assay of cAMP accumulation in human ASMC. This model also incorporates the observed effects of PMA and pertussis toxin on LPA regulation of cAMP accumulation. Which, if any, of these signaling pathways is responsible for the adaptive sensitization of cAMP accumulation that is observed after pretreatment and removal of LPA remains to be determined.

Results from the present study suggest that LPA-induced activation of protein kinase C is not likely to be the mechanism of sensitization. Protein kinase C activation with PMA did not mimic the LPA-induced sensitization of isoproterenol stimulation, so there is little reason to postulate protein kinase C mediation. Although protein kinase C activation did mimic the LPA-induced sensitization of forskolin stimulation, two lines of evidence argue against protein kinase C mediation of the LPA effect. First, LPA was able to induce essentially the same fold sensitization of forskolin stimulation in the absence or presence of maximally sensitizing concentrations of PMA. This suggests that PMA and LPA activate different pathways to induce sensitization. Second, LPA was able to induce essentially the same fold sensitization of forskolin stimulation after down-regulation of protein kinase C as in control cells.

In summary, our results suggest that LPA activates both the G_i and G_q signal transduction pathways in human ASMC. The pertussis toxin-sensitive effects are those mediated via G_i, and the pertussis toxin-insensitive effects may be mediated via G_q. Acute activation of these pathways leads to inhibition of forskolin stimulation and enhancement of isoproterenol stimulation of the type 2 isozyme of adenylyl cyclase postulated to predominate in these cells. Pretreatment of these cells with LPA induces sensitization of the activity of adenylyl cyclase, perhaps, by both pertussis toxin-sensitive

and -insensitive pathways. Activity of the adenylyl cyclase in these cells is also modulated by protein kinase C activation.

Recent studies in our laboratory have identified effects of LPA on ASMC growth and function that are of potential physiological and/or pathological significance. LPA alone stimulates mitogenesis of human ASMC (33), and it synergistically enhances the mitogenic response to epidermal growth factor (34). LPA also enhances methacholine-induced contraction and inhibits isoproterenol-induced relaxation of tracheal ring segments (35). Thus, elevated levels of LPA, or enhanced sensitivity of signaling pathways to modulation by LPA, could contribute to both the smooth muscle cell hyperplasia and the enhanced contractile sensitivity of airways observed in asthma and other airway diseases. Both of these effects would contribute to decreased airflow and ventilation. A better understanding of how LPA release, function, and metabolism are regulated in the lung and of the signaling pathways involved in the effects of LPA may thus lead to new information that will be of clinical significance.

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