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Mitogenic Effects of Cytokines on Smooth Muscle Are Critically Dependent on Protein Kinase A and Are Unmasked by Steroids and Cyclooxygenase Inhibitors

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ABSTRACT

Excessive smooth muscle growth occurs within the context of inflammation associated with certain vascular and airway diseases. The inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) have been shown previously to inhibit mitogen-stimulated smooth muscle growth through a mechanism presumed to be dependent on the induction of cyclooxygenase-2, prostaglandins, and activation of the cAMP-dependent protein kinase (PKA). Using both molecular and pharmacological strategies, we demonstrate that the mitogenic effects of IL-1β and TNF-α on cultured human airway smooth muscle (ASM) cells are tightly regulated by PKA activity. Suppression of induced PKA activity by either corticosteroids or cyclooxygenase inhibitors converts the cytokines from inhibitors to enhancers of mitogen-stimulated ASM growth, and biological variability in the capacity to activate PKA influences the modulatory effect of cytokines. Promitogenic effects of IL-1β are associated with delayed increases in p42/p44 and phosphoinositide-3 kinase activities, suggesting a role for induced autocrine factors. These findings suggest a mechanism by which mainstream therapies such as corticosteroids or cyclooxygenase inhibitors could fail to address or exacerbate the pathogenic smooth muscle growth that occurs in obstructive airway and cardiovascular diseases.

Airway smooth muscle (ASM) hyperplasia is a major feature of the airway remodeling associated with chronic asthma. Increased ASM mass contributes to increased fixed airway obstruction and, by altering airway geometry, exacerbates the reduction in airway lumen size caused by constricting agents (Deshpande and Penn, 2006). Despite the growing appreciation of ASM hyperplasia and its role in asthma pathogenesis and severity, surprisingly little is known regarding what causes it and how it should be addressed therapeutically.

Recent studies using primary cultures of ASM cells have identified various agents capable of promoting ASM proliferation and have provided insight into mitogenic signaling events. Numerous growth factors and certain G protein-coupled receptor agonists stimulate ASM cell proliferation (Billington and Penn, 2003). It is noteworthy that levels of these agents have been shown to be elevated in the airways of patients with asthma (Billington and Penn, 2003; Hirst et al., 2004), suggesting a mechanism by which airway inflammation associated with asthma can promote increased ASM mass in vivo. However, airway inflammation is also characterized by increased production of numerous cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Although IL-1β and TNF-α by themselves tend to be weak mitogens for ASM, they have been shown to significantly inhibit proliferation stimulated by several mitogenic agents (Belvisi et al., 1998; Pascual et al., 2001; Billington and Penn, 2003; Hirst et al., 2004). It is widely assumed but has never been directly demonstrated that the antimitogenic effect of IL-1β and TNF-α is mediated via cAMP-dependent

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ABBREVIATIONS: ASM, airway smooth muscle; Bis I, bisindolylmaleimide I; COX-2, cyclooxygenase-2; EGF, epidermal growth factor, FLU, fluticasone; GFP, green fluorescent protein; IL-1β, interleukin-1-β; mPGES1, microsomal prostaglandin E synthase-1; PGE_2, prostaglandin E_2; PI3K, phosphoinositide-3 kinase; PKA, cAMP-dependent protein kinase; TNF-α, tumor necrosis factor-α; VASP, vasodilator-stimulated phosphoprotein; PKI, protein kinase I; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole.

protein kinase (PKA) activity, caused by the induction of autocrine PGE_2 synthesis, which stimulates G_s-coupled EP receptors coupled to cAMP generation and PKA activation (Belvisi et al., 1998; Pascual et al., 2001; Billington and Penn, 2003; Hirst et al., 2004; Guo et al., 2005).

Herein we demonstrate that not only are the antimitogenic effects of IL-1β and TNF-α mediated by PKA, but PKA plays a critical role in suppressing what is otherwise a powerful mitogenic effect of these cytokines on ASM cells. Therefore, agents capable of suppressing cytokine-induced PKA activation, such as corticosteroids or cyclooxygenase (COX) inhibitors, have the potential to enable inflammatory environments highly conducive to smooth muscle growth.

Materials and Methods

Generation of Human ASM Cultures. Human ASM cultures were generated from tracheae from unidentified donors as described in Penn et al. (2001) and were examined in the 4th to 7th passage.

Generation of Retroviral-Infected Human ASM Cultures. The generation of retroviral-infected human ASM cultures was performed according to the methods described by Guo et al. (2005). Retrovirus for expression of GFP-, PKI-GFP-, and RevAB-GFP- expressing lines was produced by transfecting GP2–293 cells with pLNCX2-GFP, pLNCX2-PKI-GFP, or pLNCX2-RevAB-GFP, each with pVSV-G Vector, which encodes the pantropic (VSV-G) envelope protein. Supernatants were harvested 48 h after transfection and used to infect human ASM cultures. Infected cells typically exhibited >70% GFP expression within 48 h (direct visualization by fluorescent microscopy), and selection to homogeneity with 250 μg/ml G418 was rapid (7 days). In direct comparison of GFP-expressing cells with naive ASM cells from which the GFP (and PKI-GFP and RevAB-GFP) cultures were derived, results were similar (see below), demonstrating that retroviral infection per se did not affect outcomes.

Immunoblot analyses. Immunoblot analyses of time-dependent effects on phospho-Thr202/Tyr204p42/p44, phospho-S473Akt, vasodilator-stimulated phosphoprotein (VASP), COX-2, and β-actin were performed as in Billington et al. (2005) and Guo et al. (2005), using the referenced primary antibodies and infrared-conjugated secondary antibodies with detection by the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE).

[^{3}H]Thymidine Incorporation in Human ASM Cultures. [^{3}H]Thymidine incorporation in human ASM cultures was assessed according to the methods of Billington et al. (2005). In brief, cells were grown in 24-well plates to near confluence and then serum-starved in 0.1% bovine serum albumin for 24 h. Cells were pretreated with vehicle, 10 nM fluticasone propionate (FLU), or 1 μM PGE_2 for 30 min and then stimulated with vehicle, 10 nM EGF, 20 U/ml IL-1β, 10 ng/ml TNF-α, or EGF plus either 10 ng/ml TNF-α, 0.2 to 20 U/ml IL-1β, or combined 2 U/ml IL-1β+ 1 ng/ml TNF-α. After 16 h of stimulation, cells were labeled with 3.0 μCi [*methyl*-^{3}H]thymidine (1 μCi/ml) and incubated for an additional 24 h. Cells were then washed with phosphate-buffered saline, lysed with 20% trichloroacetic acid, aspirated onto filter paper, and counted in scintillation vials.

Cell Proliferation Assays. Cell proliferation assays were performed as described previously (Krymskaya et al., 2000) by growing ASM cells in six-well plates (triplicate wells per condition) to near confluence while growth-arresting and stimulating as described above for analyses of thymidine incorporation. After 48-h stimulation, cells were harvested, and viable cells were counted with an automated cell counter (ViCell; Beckman Coulter, Fullerton, CA).

Statistical Analysis. Data are presented as mean ± S.E. values from n experiments, in which each experiment was performed using a different culture derived from a unique donor. Individual data points from a single experiment were calculated as the mean value from three or six replicate observations for [^{3}H]thymidine incorporation assays or from three replicates for cell proliferation assays. For immunoblot analyses, band intensities representing signals from secondary antibody blots conjugated with infrared fluorophores were visualized and directly quantified using the Odyssey Infrared Imaging System (LI-COR). These values were normalized to values determined for β-actin and compared among stimuli and experimental groups. Statistically significant differences among groups were assessed by either analysis of variance with Fisher's protected least significant difference post hoc analysis (Statview 4.5; Abacus Concepts, Berkeley, CA) or by t test for paired samples, with p values <0.05 sufficient to reject the null hypothesis.

Results

Previous studies have demonstrated that IL-1β (and more variably, TNF-α) treatment of naive human ASM cultures reduces growth factor- and serum-stimulated increases in DNA synthesis and cell number (Belvisi et al., 1998; Pascual et al., 2001; Vlahos et al., 2003). To directly examine the role of PKA in this antimitogenic effect, we generated human ASM cultures stably expressing GFP chimeras of two different inhibitors of PKA (PKI and RevAB) and GFP-expressing control cultures, with each set of cultures derived from the same (naive) donor cells (Guo et al., 2005). Effective inhibition of PKA activation in PKI-GFP and RevAB-expressing cells was demonstrated previously in Guo et al. (2005) by significant inhibition of both PGE_2- and IL-1β-stimulated phosphorylation of the intracellular PKA substrate VASP.

In GFP-expressing ASM cells, IL-1β (20 U/ml) alone did not significantly affect [^{3}H]thymidine incorporation but significantly inhibited EGF-stimulated increases (Fig. 1A). In cells expressing PKI-GFP or RevAB-GFP, IL-1β alone was a more effective mitogenic stimuli, but most strikingly, it no longer inhibited but greatly increased the mitogenic effect of EGF. Similar effects on ASM cell number were observed (Fig. 1B).

If the principal source of cytokine-stimulated PKA activity responsible for the suppressive effect on EGF-stimulated growth is COX-2-derived PGE_2, we hypothesized that agents capable of inhibiting the induction of COX-2 protein or activity or inhibiting other enzymes important in cytokine-induced PGE_2 production (Pascual et al., 2006) would have similar effects. This proved to be the case, because pretreatment of naive ASM cells with indomethacin (an inhibitor of COX activity), SB203580 or bisindolylmaleimide I (Bis I) (inhibitors of p38 and protein kinase C, respectively) shown previously to inhibit induction of COX-2 and mPGES1 by IL-1β in ASM cells (Pascual et al., 2006) also increased IL-1β-stimulated growth and converted IL-1β from an inhibitor to an enhancer of EGF-stimulated growth (Fig. 2A). Pretreatment with the corticosteroid FLU, known to be a strong suppressor of cytokine-induced prostanoid synthesis (Vlahos and Stewart, 1999; Pascual et al., 2006), also caused IL-1β to enhance EGF-stimulated growth. The magnitude of effects of indomethacin, SB203580, Bis I, or FLU corresponded to the efficacy of each inhibitor in inhibiting EGF + IL-1β-induced PKA activation, which was determined by characterizing the reversal of the shift of the PKA substrate VASP from the 50-kDa species to the 46-kDa species (Fig. 2, C and D). Indomethacin, SB203580, and FLU proved to be strong inhibitors of induced PKA activity, whereas Bis I was effective but less so, consistent with the lesser ability of Bis I to augment EGF + IL-1β-stimulated thymidine incorporation.

Unlike the other inhibitory strategies, FLU inhibited the weak mitogenic effect of IL-1β alone (Fig. 2B). Pretreatment of cells with dexamethasone had similar effects to those of FLU (data not shown).

To investigate whether the effects of FLU and direct PKA inhibition share a similar mechanistic basis, we compared the effects of FLU with those of PKI-GFP expression and examined the combined effects of both FLU pretreatment and PKI-GFP expression. EGF+IL-1β-stimulated growth was similar in GFP- and PKI-GFP-expressing cells pretreated with FLU (Fig. 3). However, FLU pretreatment of PKI-GFP cells caused a significant reduction of EGF+IL-1β-

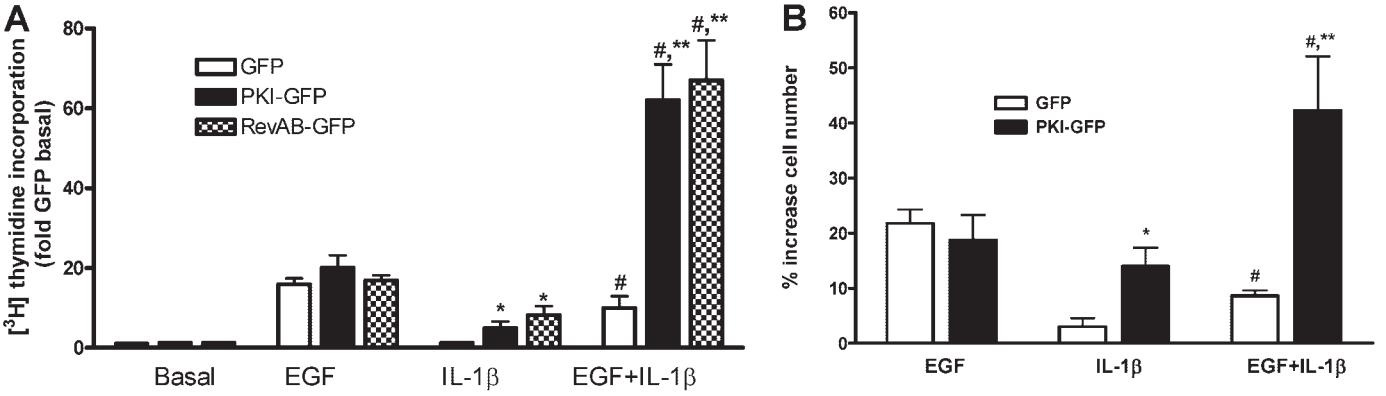

Fig. 1. PKA inhibition promotes mitogenic effects of IL-1β. Effect of PKA inhibition via heterologous expression of PKA inhibitors PKI or RevAB on IL-1β-, EGF-, and EGF + IL-1β-stimulated [3H]thymidine incorporation (A) and cell proliferation (B). Bars represent mean ± S.E. values; $n = 11$ (A) and $n = 4$ (B). *, $p < 0.05$ IL-1β PKI-GFP or RevAB-GFP versus IL-1β GFP; #, EGF + IL-1β (all groups) versus EGF GFP; **, EGF + IL-1β PKI-GFP or RevAB-GFP versus EGF + IL-1β GFP.

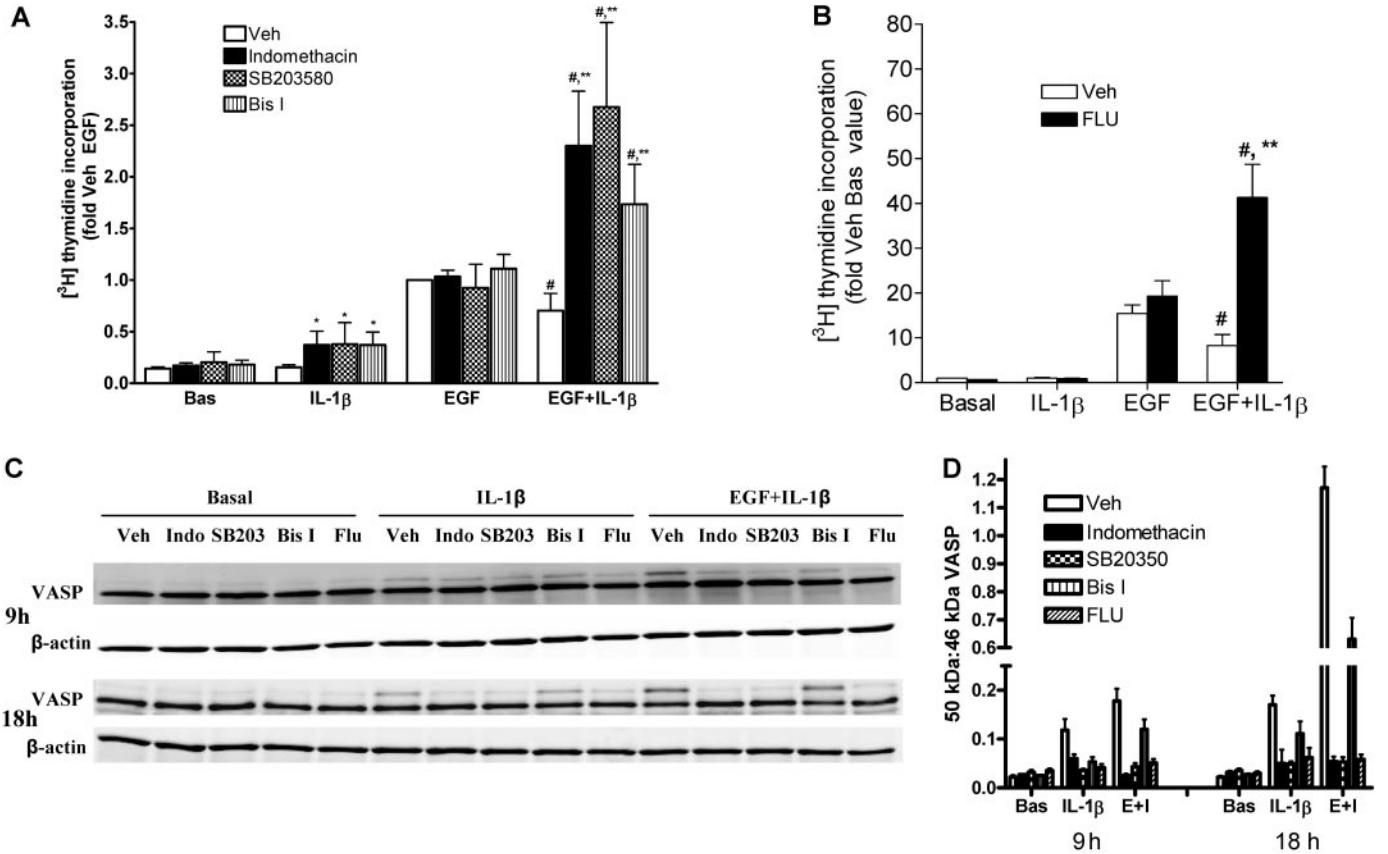

Fig. 2. Multiple inhibitors of IL-1β-induced PGE_2 synthesis promote a mitogenic effect of IL-1β in ASM cells. A, effect of 30-min pretreatment with inhibitors of cyclooxygenase activity (1 μM indomethacin), p38 (1 μM SB203580), or cPKC isoforms (3 μM Bis I) on EGF-, IL-1β-, or EGF + IL-1β-stimulated [3H]thymidine incorporation in naive human airway smooth muscle cultures. B, effect of 30-min pretreatment with 10 nM FLU on ASM [3H]thymidine incorporation. Bars represent mean ± S.E. values; $n = 4$ to 6 (A), $n = 12$ (B). *, $p < 0.05$ IL-1β indomethacin, SB203580, Bis I, or FLU versus IL-1β GFP; #, EGF + IL-1β (all groups) versus EGF GFP; **, EGF + IL-1β indomethacin, SB203580, Bis I, and FLU versus EGF + IL-1β GFP. C, representative immunoblot assessing shifts in VASP protein mobility as a readout for PKA activation promoted by 9- and 18-h treatment with the IL-1β or EGF + IL-1β in cells pretreated with either vehicle, indomethacin, SB203580, Bis I, or FLU. D, the relative ratio of the 50- and 46-kDa VASP band intensities was normalized to the corresponding values for β-actin. Data shown represent mean + S.E. values from four independent experiments.

stimulated growth. These results suggest that suppression of induced PKA activity is the principal and shared mechanism mediating the effects of FLU and PKI-GFP, yet FLU is able to suppress a contributory mechanism promoted by PKA inhibition.

In a subset of experiments in which the effects of PKA inhibition on the regulatory effects of IL-1β and TNF-α were compared, PKI-GFP expression and FLU pretreatment was shown to similarly increase EGF-stimulated [^{3}H]thymidine incorporation in cells costimulated with TNF-α or IL-1β (Fig. 4). However, in GFP-expressing cells, TNF-α did not significantly inhibit EGF-stimulated [^{3}H]thymidine incorporation. When EGF-stimulated cells were costimulated with combined IL-1β and TNF-α, both at low concentrations (2 U/ml and 1 ng/ml, respectively), the effect was comparable with that induced by 20 U/ml IL-1β alone.

To further explore the relationship between PKA activity and the mitogenic effects of IL-1β, the dose-dependent effect of IL-1β on EGF-stimulated ASM growth and associated mitogenic signaling events was examined. In both naive and GFP-expressing cells, 20 U/ml IL-1β inhibited EGF-stimulated growth, 2 U/ml IL-1β caused either no change or a slight increase in EGF-stimulated growth, and 0.2 U/ml IL-1β significantly enhanced EGF-stimulated growth (Fig. 5A). The increasing promitogenic effect with progressively lower concentrations of IL-1β was associated with a diminishing induction of both COX-2 protein and PKA activity, the latter paralleled by progressively lower levels of phosphorylated (50 kDa) VASP (Fig. 5B). In PKI-GFP-expressing cultures, in which induction of VASP phosphorylation was nearly abolished, each of the concentrations of IL-1β was highly efficacious in augmenting EGF-stimulated growth, with only minimal differences in the effect observed.

We have demonstrated previously important roles for p42/p44, PI3K, and p70S6K activities in the regulation of ASM growth (Orsini et al., 1999; Krymskaya et al., 2000; Billington et al., 2005; Kong et al., 2006). In naive and GFP-expressing cells, the large COX-2 induction and PKA activation stimulated by 18-h treatment with EGF + 20 U/ml IL-1β were associated with either a slight inhibition or no change in p42/p44 and Akt phosphorylation (Akt phosphorylation serves as a readout for PI3K activation; Billington et al., 2005; Kong et al., 2006) (Fig. 5B). However, lower concentrations of IL-1β promoted an increase in Akt and, to a slightly lesser extent, p42/p44 phosphorylation. Phospho-p70S6K levels were low and could not be reliably quantified at time points exceeding 6 h (data not shown). In PKI-GFP-expressing cells, all concentrations of IL-1β enhanced EGF-stimulated p42/p44 and Akt phosphorylation.

Analysis of the time-dependent regulation of p42/p44 and Akt phosphorylation revealed that at early time points up to 9 h, 20 U/ml IL-1β increased EGF-stimulated Akt phosphorylation and caused either no change or a slight increase in p42/p44 phosphorylation (Fig. 6). These patterns were similar in PKI-GFP-expressing cells. FLU treatment had a slight inhibitory effect on Akt phosphorylation in both GFP- and PKI-GFP-expressing, yet the relative effect of IL-1β was retained. On the other hand, FLU tended to augment p42/p44 phosphorylation stimulated by EGF and EGF + IL-1β in both cell lines. By 18 h, and coinciding with higher levels of

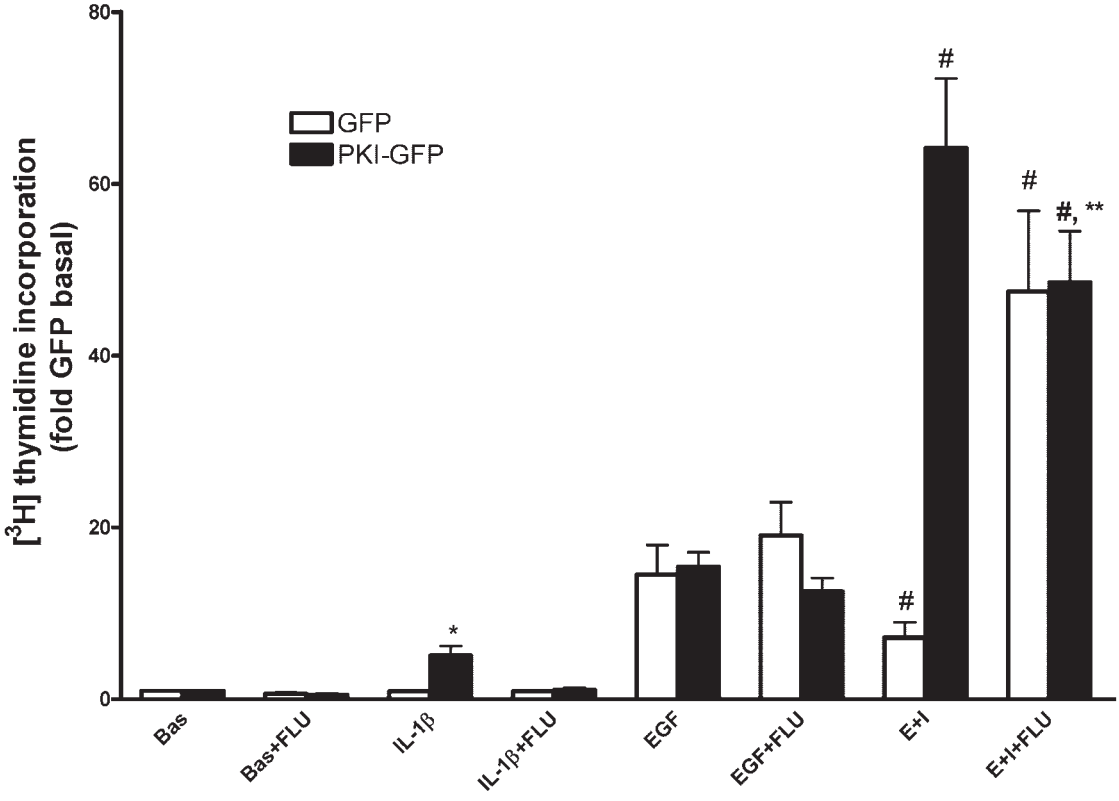

Fig. 3. Glucocorticoid pretreatment (FLU) promotes similar promitogenic effects to those induced by PKA inhibition. Effect of FLU pretreatment (10 nM, 30 min) on [^{3}H]thymidine incorporation in GFP- and PKI-GFP-expressing ASM cells stimulated with IL-1β, EGF, or EGF + IL-1β for 18 h. Bars represent mean ± S.E. values; $n = 12$. *, $p < 0.05$ IL-1β PKI-GFP versus IL-1β GFP; #, EGF + IL-1β (GFP or PKI-GFP) and EGF + IL-1β + FLU (GFP or PKI-GFP) versus EGF GFP; **, EGF + IL-1β + FLU PKI-GFP versus EGF + IL-1β PKI-GFP.

COX-2 induction and PKA activity, IL-1β no longer augmented EGF-stimulated phospho-p42/p44 and Akt levels in GFP-expressing cells, in some lines having a slight inhibitory effect. However, at 18 h IL-1β retained its ability to promote higher levels of Akt and p42/p44 phosphorylation in cells treated with FLU or expressing PKI. These time- and line-dependent regulatory effects on Akt and p42/p44 phosphorylation were inversely related to the level of PKA activity indicated by the shift in VASP mobility.

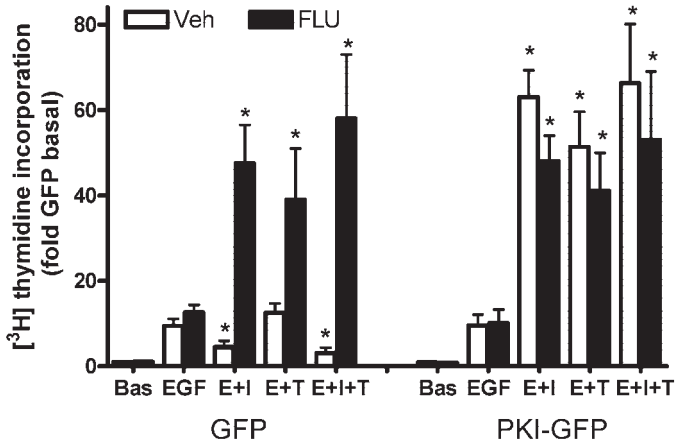

Fig. 4. PKA inhibition, FLU pretreatment regulate modulation of EGF-stimulated thymidine incorporation by TNF-α. GFP or GFP-PKI were pretreated with vehicle or 10 nM FLU then stimulated with EGF (10 ng/ml) ± IL-1β (I; 20 U/ml), TNF-α (T; 10 ng/ml), or IL-1β + TNF-α (I+T; 2 U/ml and 1 ng/ml, respectively). Bars represent mean ± S.E. values; $n = 4$ to 5. *, $p < 0.05$, EGF + IL-1β and EGF + IL-1β + TNF-α (GFP or PKI-GFP) and EGF + IL-1β + FLU, EGF + TNF-α + FLU, or EGF + IL-1β + TNF-α + FLU (GFP or PKI-GFP) versus EGF (GFP or PKI-GFP).

Consistent with a role of PKA in suppressing the otherwise mitogenic effect of IL-1β on EGF-stimulated growth, co-stimulation of cultures with 1 μM PGE_2 was able to inhibit the large increase in EGF + IL-1β-stimulated [^{3}H]thymidine incorporation conferred by FLU in naive and GFP-expressing cells, but not in PKI-GFP expressing cells (Fig. 7, A–C). It is interesting that exogenous PGE_2 significantly increased [^{3}H]thymidine incorporation in PKI-GFP cells stimulated by EGF. This result suggests that under conditions of PKA inhibition, EP1 or EP3 receptor-dependent mitogenic signaling can emerge to cooperate with that stimulated by EGF (Billington et al., 2005; Kong et al., 2006). Further analysis of the dose-dependent effect of PGE_2 on FLU-treated EGF + IL-1β-stimulated cells demonstrates the high potency of PGE_2 in inhibiting thymidine incorporation under these conditions (Fig. 7D).

Finally, an interesting observation afforded by the use of cultures generated from numerous donors was the degree of biological variability exhibited in the regulatory effect of IL-1β on EGF-stimulated growth. Analysis of data derived from 13 different (naive) ASM cultures revealed considerable variability in the inhibitory effect of IL-1β on EGF-stimulated [^{3}H]thymidine incorporation (Fig. 8A). Indeed, in 2 of 13 cultures, 20 U/ml IL-1β caused an increase in EGF-stimulated [^{3}H]thymidine incorporation. To explore whether this variability could be attributed to a variation in the induction of COX-2 or PKA activation, lysates for those cultures examined for COX-2 protein and VASP phosphorylation induced after 18-h stimulation were run on a single gel, and an

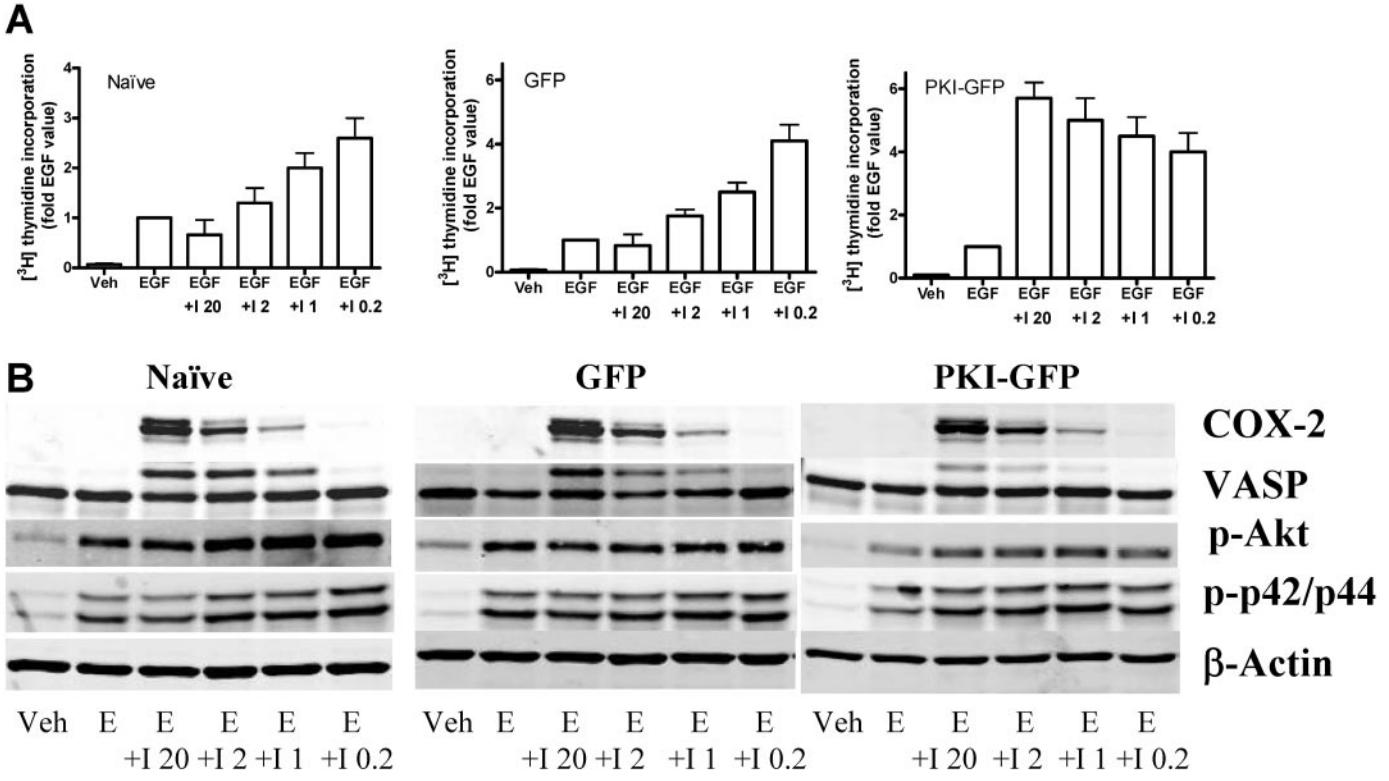

Fig. 5. Dose-dependent effect of IL-1β on ASM growth is associated with the induction of COX-2 expression and PKA activation and inhibition of PI3K and p42/p44 pathways. Effects of 0.2 to 20 U/ml IL-1β on EGF-stimulated [^{3}H]thymidine incorporation (A) and induction of COX-2 expression, PKA activation (readout as VASP phosphorylation), and phosphorylation of Akt (readout for PI3K activity), and p42/p44 (B) in naive, GFP-, and PKI-GFP-expressing cells. Bars represent mean ± S.E. values from three independent experiments using three different cultures.

immunoblot was generated. Variability in both COX-2 and VASP phosphorylation was observed, and correlation analysis demonstrates a relationship between the outcomes and the inhibitory effect of IL-1β on EGF-stimulated growth (represented as the ratio of thymidine incorporation stimulated by EGF+IL-1β:EGF) (Fig. 8, B–D).

Discussion

Results from the present study demonstrate the critical role of PKA in determining the modulatory effect of the cytokines IL-1β and TNF-α on ASM growth, thereby revealing a mechanism by which clinically relevant agents could promote smooth muscle growth in vivo and exacerbate disease. Several studies have reported inhibitory effects of IL-1β or TNF-α on ASM growth stimulated by either growth factors, G protein-coupled receptor agonists, or serum (Belvisi et al., 1998; Pascual et al., 2001; Vlahos et al., 2003). The magnitude of the inhibitory effects varies among studies and may be attributed to differences in the species of ASM and culture conditions and, as suggested by the present study, variability among the cultures themselves. Combined treatment with IL-1β and TNF-α, arguably the condition more relevant to the inflamed airway, has an even greater inhibitory effect than either agent alone (Belvisi et al., 1998; Pascual et al., 2001).

IL-1β and (to a lesser extent) TNF-α can induce COX-2 protein and PGE_2 generation in ASM cells, and cotreatment with serum or EGF greatly increases this induction (Pascual et al., 2001, 2006). For this reason, and given the observation that exogenous PGE_2 inhibits ASM growth, COX-2-dependent PGE_2 production has been assumed to mediate inhibition of ASM growth by these cytokines. Because PGE_2 can stimulate cAMP production in ASM cells, it is in turn widely assumed that PKA promotes the antimitogenic signaling generated by long-term IL-1β and TNF-α treatment. However, although a role for COX-2 in the antimitogenic effects of cytokines has sufficient empirical basis, no evidence directly implicating PKA exists. The failure to implicate PKA stems

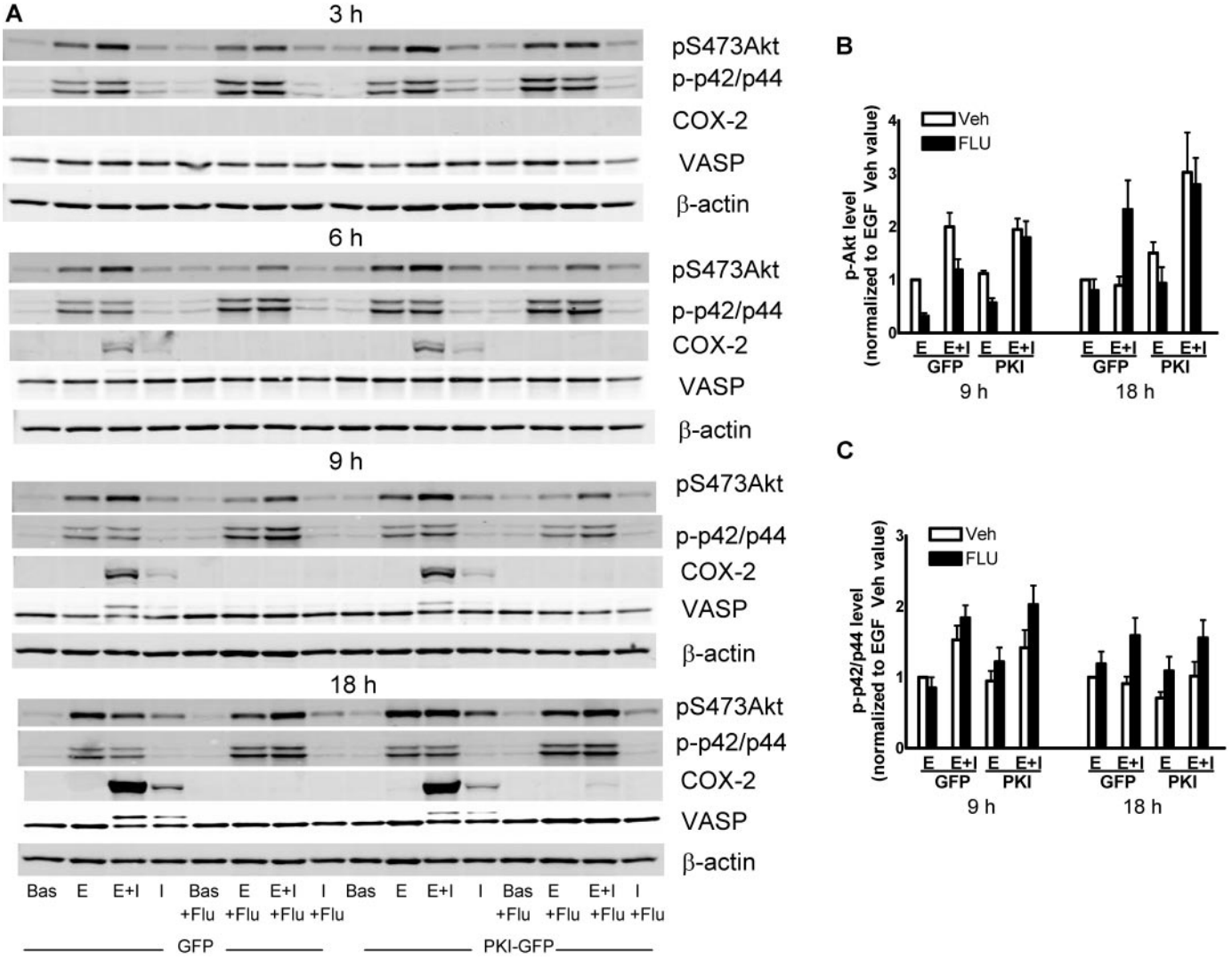

Fig. 6. FLU pretreatment or expressing PKI-GFP expression determines IL-1β effects on late-phase Akt and p42/p44 phosphorylation. A, GFP- and PKI-GFP-expressing ASM cells were pretreated with FLU (10 nM, 30 min) and then stimulated with EGF, IL-1β, or EGF + IL-1β for 3, 6, 9, or 18 h. Cell lysates were subjected to immunoblotting to evaluate pS473Akt, pT202/Y204p42/p44, COX-2, VASP, and β-actin expression. B and C, band intensities for p-Akt and p-p42/p44 for the 9- and 18-h time points were quantified and normalized to corresponding values for β-actin. Data shown represent mean + S.E. values from four independent experiments.

from difficulties in both characterizing and directly inhibiting PKA activity in primary cells. Although pharmacological agents known to inhibit PKA activity in vitro are frequently used as a means to directly inhibit PKA activity in intact cells, we have demonstrated recently that many of the most commonly used pharmacological PKA inhibitors are not only ineffective in ASM cultures but also promote problematic nonspecific effects (Penn et al., 1999; Guo et al., 2005).

On the other hand, heterologous expression of PKI, which functions as a substrate mimetic, or RevAB, a mutant PKA regulatory subunit incapable of binding cAMP (Correll et al., 1989), successfully inhibits the activation of PKA by multiple stimuli in ASM (Guo et al., 2005). In PKI-GFP- or RevAB-GFP-expressing ASM cells, IL-1β and TNF-α acquired the ability to stimulate ASM growth by themselves and were converted from inhibitors to enhancers of EGF-stimulated growth. The role of PKA in dictating the mitogenic effect of cytokines is further suggested by the association of inhibition of COX-2 and PGE_2 induction via multiple strategies with a similar reversal of the growth inhibition by IL-1β.

The ability of corticosteroids or COX inhibition to convert cytokines from inhibitors to enhancers of mitogen-stimulated growth has been reported previously for both airway (Vlahos and Stewart, 1999; Vlahos et al., 2003) and vascular (Libby et al., 1988) smooth muscle, although the mechanistic basis of these effects was not determined. Vlahos and Stewart (1999) reported that pretreatment of human ASM with either indomethacin or dexamethasone caused combined IL-1α and TNF-α treatment to augment (which otherwise inhibited) [^{3}H]thymidine incorporation stimulated by basic fibroblast growth factor. Libby et al. (1988) reported that indomethacin pretreatment of human vascular smooth muscle cells causes IL-1α to augment (rather than inhibit) [^{3}H]thymidine incorporation stimulated by fetal calf serum. Although in both studies these promitogenic effects of cyclooxygenase inhibition/steroid treatment were associated with inhibition of prostaglandin induction, neither study examined the specific role of PKA in this reversal.

In the current study, the role of PKA inhibition in the ability of steroids and COX inhibition to render IL-1β a powerful enhancer of EGF-stimulated ASM growth is strongly suggested by the inability of FLU pretreatment to further enhance [^{3}H]thymidine incorporation stimulated by EGF + IL-1β in PKI-GFP expressing cells. Although it is likely that steroids affect numerous factors that regulate ASM growth that are distinct from those affected by PKA inhibition, the largely redundant effect of FLU treatment and PKA inhibition on IL-1β modulation of EGF-stimulated ASM growth strongly suggests that PKA inhibition by FLU, a consequence of suppression of prostanoid induction, contributes significantly to the promitogenic effect observed.

By virtue of the ability to screen multiple cultures of ASM, each derived from a different donor, we discovered considerable variability in the modulatory effect of IL-1β. In fact, in two cultures, 20 U/ml IL-1β significantly increased EGF-stimulated growth. This variability in regulating growth was associated with similar variability in intracellular PKA activity. Multiple pro- and antimitogenic signals are probably involved in the regulation of growth by combined growth factor and cytokine stimulation. Promitogenic signaling in

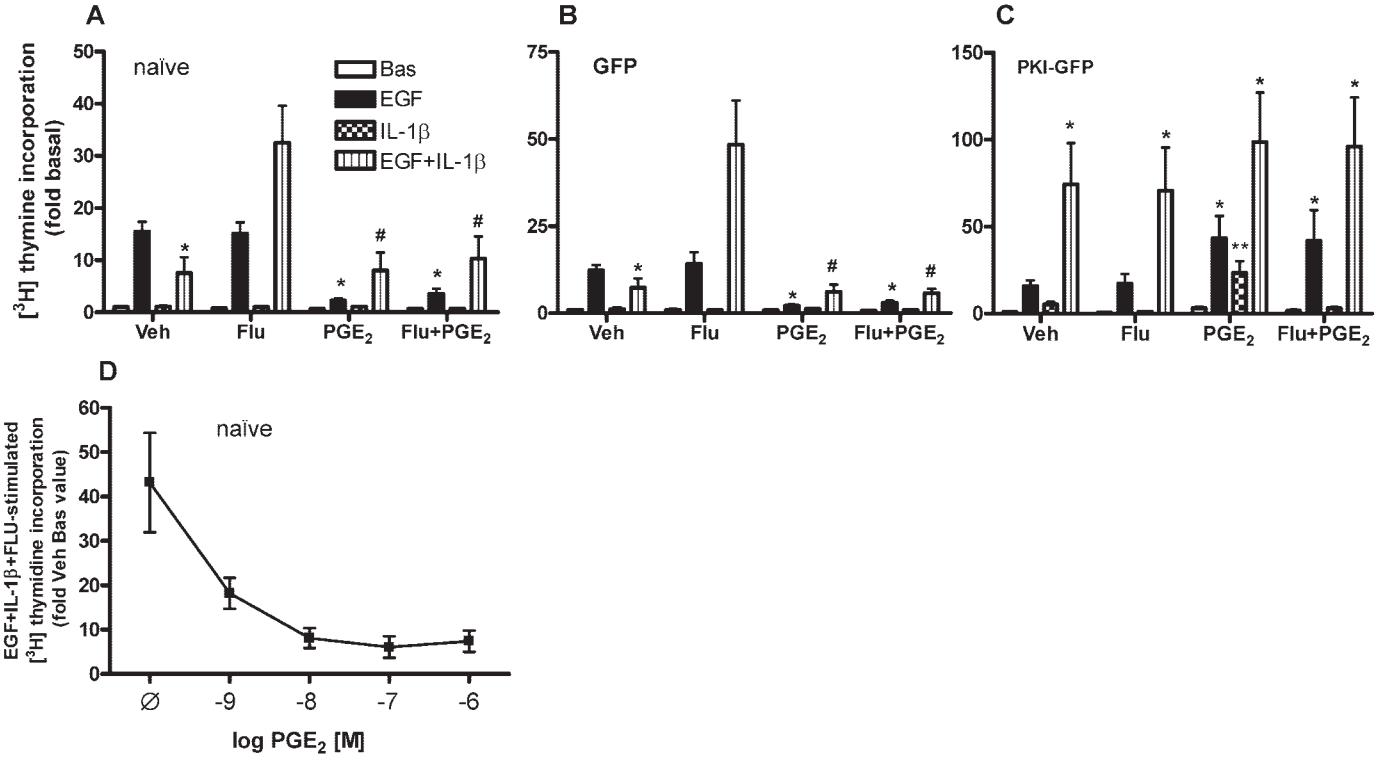

Fig. 7. Exogenous PGE_2 reverses mitogenic effect of fluticasone treatment but not direct PKA inhibition on EGF + IL-1β-stimulated ASM cells. Naive (A), GFP- (B), or PKI-GFP- (C) expressing cells were pretreated with vehicle or 10 nM FLU ± 1 μM PGE_2 before stimulation with the indicated agents. Cells were subsequently harvested for analysis of [^{3}H]thymidine incorporation as described under *Materials and Methods*. Bars represent mean ± S.E. values, $n = 7$. *, $p < 0.05$ indicated value versus vehicle-pretreated EGF value; #, indicated value versus EGF + IL-1β FLU value; **, IL-1β PGE_2 PKI-GFP versus IL-1β vehicle PKI-GFP. D, cells were pretreated with vehicle or 10 nM FLU ± PGE_2 ranging in concentration from 1 nM to 1 μM, stimulated with EGF + IL-1β, and analyzed for [^{3}H]thymidine incorporation as above; $n = 4$.

ASM includes the p42/p44, PI3K, and p70S6K pathways (Hirst et al., 2004; Deshpande and Penn, 2006). However, these pathways are also important in inducing enzymes (cPLA2, COX-2, and mPGES1) responsible for PGE_2 synthesis and antimitogenic PKA activation (Pascual et al., 2001, 2006; R. M. Pascual and R. B. Penn, unpublished observations). Differences in the capacity of IL-1β and TNF-α to induce PKA activity could stem from variability in the expression or activity of numerous proteins linking IL-1β and TNF-α with PKA activation—IL-1β or TNF-α receptors, signaling molecules proximal and downstream of IL-1β/TNF-α receptors (interleukin-1 receptor-associated kinase, TNF receptor-associated factor, TNF factor receptor-associated death domain, nuclear factor-κB-inducing kinase, etc.), cytosolic regulators of cPLA2, COX-2, and mPGES1 (mitogen-activated protein kinases, PI3K isoforms, p70S6K, and nuclear factor-κB), enzymes mediating PGE_2 synthesis (cPLA2, COX-2, and mPGES1), EP2/4 receptors, the heterotrimeric G protein G_s and the downstream effector adenylyl cyclase, and finally regulatory and catalytic subunits of PKA, as well as additional molecules that regulate PKA enzymatic activity (multiple kinases) and localization (A kinase-anchoring proteins). It is conceivable that patients with asthma in whom cytokines promote minimal PGE_2 generation/PKA activation are at greater risk of inflammation-induced increases in ASM mass, do not outgrow their disease, and develop chronic asthma with a fixed obstruction component. Should our results in cultured cells be relevant to the in vivo condition, corticosteroid therapy could further compound this problem by effectively inhibiting the induction of prostaglandins and PKA activity and further enabling promitogenic signaling by cytokines. Although it is true that inhaled steroids are effective in reducing inflammatory cell accumulation and cytokine levels in the lung, they are frequently administered during ongoing inflammation, when cytokine levels are already elevated. Studies to date have only examined the effects of prophylactic corticosteroid administration on features of airway remodeling, using mouse or rat models in which allergic inflammation was induced by short-term sensitization and challenge with ovalbumin (Vanacker et al., 2002a,b; Miller et al., 2006). Thus, additional studies examining more clinically relevant conditions are required to clarify the regulatory effects of corticosteroids on ASM growth in vivo.

A similar regulatory role of PKA in vascular smooth muscle growth that occurs during inflammation-driven injury and repair of the vascular wall could also greatly influence various forms and consequences of vascular disease. Lipid deposition in the vessel wall and subsequent infiltration of the intima by inflammatory cells are known to contribute to initial plaque formation. Inflammatory cells in the environment of the intima expose vascular smooth muscle cells to

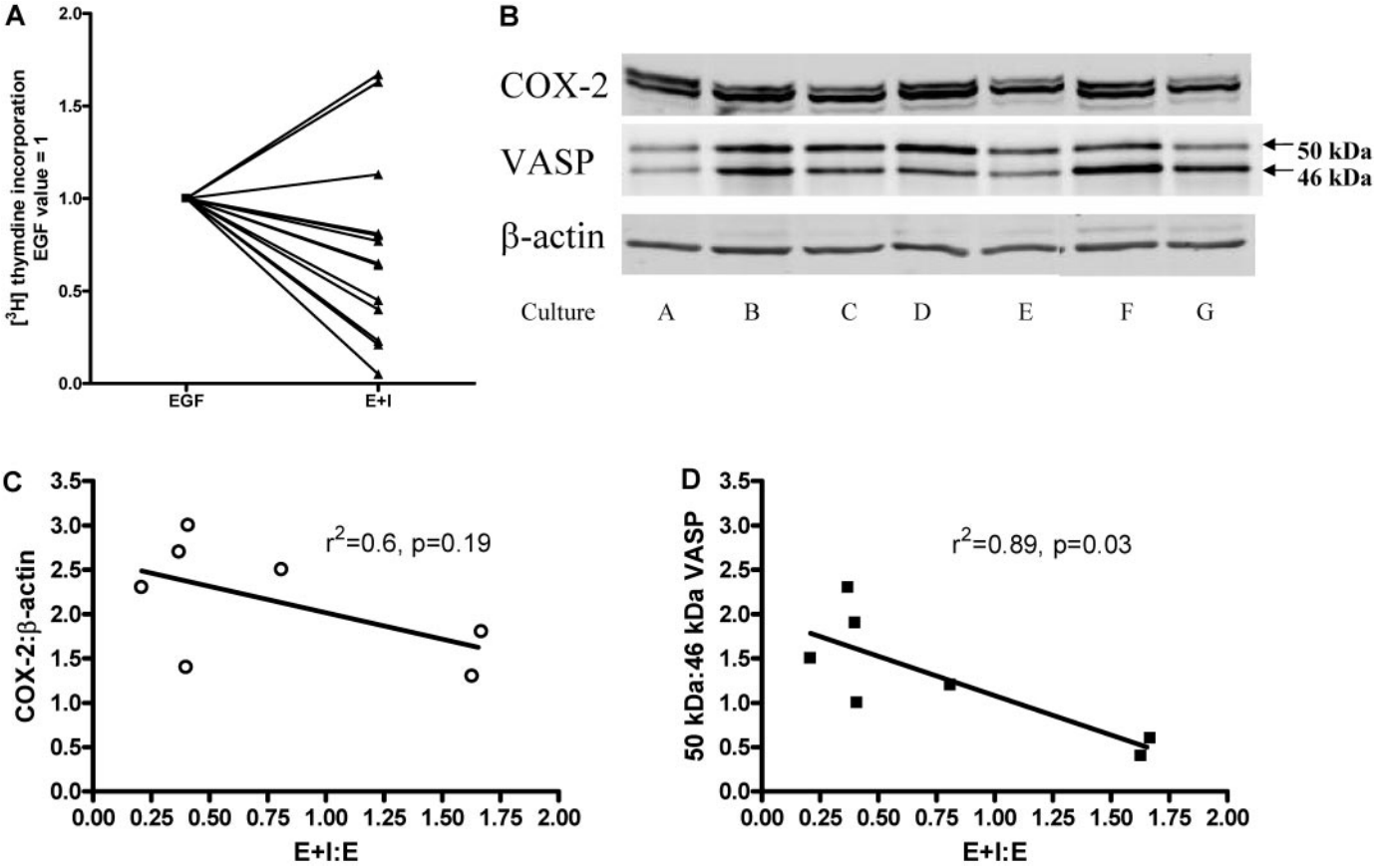

Fig. 8. Variability in the effect of IL-1β on EGF-stimulated growth in ASM cells is correlated to the induction of COX-2 expression and PKA activation. A, regulatory effect of IL-1β on EGF-induced [^{3}H]thymidine incorporation in naive ASM cells. Assay performed as described under *Materials and Methods*, $n = 13$. B, ASM cells derived from seven different donors were stimulated with EGF + IL-1β for 18 h. Cell lysates were subjected to immunoblotting to assess COX-2 expression and VASP shift/phosphorylation. C and D, correlation analysis between the ability of IL-1β to regulate EGF-stimulated ASM growth (expressed as ratio of [^{3}H]thymidine incorporation stimulated by EGF + IL-1β to EGF) and its ability to induce COX-2 expression (normalized to β-actin) (C) or PKA activation (expressed as ratio of 50:46-kDa VASP; D), $n = 7$.

cytokines, growth factors, and other vasoactive substances and leads to phenotypic modulation of the smooth muscle. The initial vascular smooth muscle migration, proliferation, and accumulation at the site of the lesion are fundamental processes leading to atherosclerotic plaque formation that contributes to myocardial infarction and stroke pathogenesis (Ross and Glomset, 1973; Campbell and Campbell, 1994; Bornfeldt and Krebs, 1999). Under these conditions, the clinical use of cyclooxygenase inhibitors could create an environment favoring exaggerated smooth muscle growth. Such growth could further destabilize the vascular wall, increasing the likelihood of further inflammation, plaque formation, and damage leading to various acute clinical events.

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