Molecular Pharmacology Fast Forward. Published on February 6, 2013 as doi:10.1124/mol.112.083493

Phosphodiesterase 4 inhibitors augment the ability of formoterol to enhance glucocorticoid-dependent gene transcription in human airway epithelial cells: a novel mechanism for the clinical efficacy of roflumilast in severe COPD

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Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
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Running Head: Roflumilast, GRE-dependent transcription and severe COPD

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Manuscript Information:

Number of Text Pages: **41**
Number of Tables: **4**
Number of Figures: **11**
Number of References: **40**
Number of words in Abstract: **250**
Number of words in Introduction: **750**
Number of words in Discussion: **1535**

ABBREVIATIONS: BEBM, bronchial epithelial cell basal medium; BEGM, bronchial epithelial cell growth medium; CD, cluster of differentiation; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CRISPLD2, cysteine-rich secretory protein LCCL (Limulus clotting factor C, Cochlin, Lgl1) domain-containing 2, DMEM, Dulbecco's modified Eagle's medium; *E*/[A], concentration-effect; EMA, European Medicines Agency; FP, fluticasone propionate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GILZ, glucocorticoid-inducible leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK 256066 (6-[[3-[(dimethylamino)-carbonyl]phenyl]sulphonyl]-4-[(3-methoxyphenyl)amino]-8-methyl-3-quinoline carboxamide); ICS, inhaled corticosteroid; HpAECs, human primary airway epithelial cells; LABA, long-acting β_2-adrenoceptor agonist; MOI, multiplicity of infection; PKA, cAMP-dependent protein kinase; PKI, cAMP-dependent protein kinase inhibitor; $p57^{kip2}$, kinase inhibitor protein 2 of 57 kDa; PDE, phosphodiesterase; RGS, regulator of G-protein signalling; SFM, serum-free medium.

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ABSTRACT *Post hoc* analysis of two phase III clinical studies found that the phosphodiesterase (PDE) 4 inhibitor, roflumilast, reduced exacerbation frequency in patients with severe chronic obstructive pulmonary disease (COPD) that were taking inhaled corticosteroids (ICS) concomitantly whereas patients not taking ICS derived no such benefit. In contrast, in two different trials, also performed in patients with severe COPD, roflumilast reduced exacerbation rates in the absence of ICS, indicating that PDE4 inhibition alone is sufficient for therapeutic activity to be realised. Given that roflumilast is recommended as an "add-on" medication to patients with severe disease that will inevitably be taking a LABA/ICS combination therapy, we tested the hypothesis that roflumilast *augments* the ability of glucocorticoids to induce genes with anti-inflammatory activity. Using a glucocorticoid response element (GRE) luciferase reporter transfected into human airway epithelial cells (both BEAS-2B cells and primary cultures), roflumilast enhanced fluticasone propionate-induced GRE-dependent transcription. Roflumilast also produced a sinistral displacement of the concentration-response curves that described the augmentation of GRE-dependent transcription by the long-acting β_2-adrenoecoptor agonist (LABA), formoterol. In BEAS-2B cells and primary airway epithelia, roflumilast interacted with formoterol in a positive cooperative manner to enhance the expression of several glucocorticoid-inducible genes that have anti-inflammatory potential. We suggest that the ability of roflumilast and formoterol to interact in this way supports the concept that these drugs together may impart clinical benefit beyond that achievable by an ICS alone, a PDE4 inhibitor alone or an ICS/LABA combination therapy. Roflumilast may, therefore, be especially effective in patients with severe COPD.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a general term that encompasses several debilitating pathologies that often co-exist, and is characterised by a progressive and largely *irreversible* decline in lung function. Persistent chronic airflow limitation, usually associated with airway collapse, edema, mucus hyper-secretion and fibrosis are present to a greater or lesser extent and accounts for the wide spectrum of disease. Typically, COPD afflicts middle aged and elderly people and is caused, predominantly, by chronic cigarette smoking (Hogg et al., 2004, Hogg, 2004).

COPD is characterised by neutrophilic inflammation of the small airways and lungs that, contrary to asthma, is relatively resistant to inhaled corticosteroids (ICS) (Barnes, 2008). Consequently, the management of COPD is hampered by a lack of disease-modifying, anti-inflammatory therapies. However, in April 2010 the European Medicines Agency recommended approval of the phosphodiesterase (PDE) 4 inhibitor, roflumilast, for the "*maintenance treatment of patients with severe COPD associated with chronic bronchitis who have a history of frequent exacerbations*" (http://www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/human/001179/WC500089626.pdf). Subsequently, in 2011, roflumilast was approved by Health Canada (www.hc-sc.gc.ca/dhp-mps/prodpharma/applic-demande/regist/reg_innov_dr-eng.php) and the United States Food and Drug Administration (www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm244989.htm). The approval of roflumilast for COPD is significant because it is believed to provide clinical benefit by suppressing inflammation (Giembycz and Field, 2010, Gross et al., 2010, Hatzelmann et al., 2010).

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) recently updated its recommendations for the treatment of stable COPD to include roflumilast as a second choice medication in high risk patients with severe, symptomatic disease (GOLD stages 3-4) (www.goldcopd.org/uploads/users/files/GOLD_Report_2011_Jan21.pdf). Thus, National and International guidelines, when revised, are likely to advocate that roflumilast be used as an "add-on" medication to patients already taking tiotropium bromide *and* a LABA/ICS combination therapy.

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Clinically, there may be a scientific rationale for using a PDE4 inhibitor in combination with an ICS. In a pooled *post hoc* analysis of two phase III studies, roflumilast reduced exacerbation frequency in a subgroup of patients with severe COPD that were taking an ICS concomitantly, whereas patients not taking an ICS derived no such benefit (Rennard et al., 2011). Roflumilast also improved lung function in patients diagnosed with COPD associated with chronic bronchitis, with or without co-existing emphysema that was greater if they had received concomitant ICS relative to placebo (Rennard et al., 2011). However, two additional phase III trials, also in severe COPD patients, found that roflumilast reduced exacerbation rates the absence of an ICS (Calverley et al., 2009), indicating that PDE4 inhibition alone may be sufficient for clinical benefit to be realised. Although the reason for this discrepancy remains unclear, the possibility that the efficacy of roflumilast and an ICS together maybe superior than either drug alone is attractive from a therapeutics standpoint. Indeed, frequent exacerbations of COPD are associated with a high level of inflammation (Perera et al., 2007) that may be more responsive to these anti-inflammatory therapies when given in combination.

Glucocorticoids exert anti-inflammatory activity principally by repressing the expression of pro-inflammatory genes (Newton et al., 2010). Two general mechanisms have been described. The most widely accepted of these is *trans*repression, in which the agonist-bound glucocorticoid receptor (GR) hinders the ability of transcription factors, such as NFκB and AP-1, to promote the transcription of pro-inflammatory genes. Recently, *trans*repression via a direct interaction of the agonist-bound GR to negative glucocorticoid response elements (GREs) was also defined (Surjit et al., 2011). However, in simple systems, glucocorticoids are often only partial inhibitors of pro-inflammatory gene transcription, implying that processes in addition to *trans*repression must be operative to explain the anti-inflammatory effects seen in *bona fide* models of inflammation (Clark, 2007, Newton et al., 2010). Indeed, there is now persuasive evidence that the induction (*trans*activation) of genes, many encoding proteins with anti-inflammatory potential, constitutes a major mechanism of glucocorticoid action (Clark, 2007, Clark and Belvisi, 2011, Newton et al., 2010).

Herein, we have used human bronchial epithelial cells as a model system to test the hypothesis that PDE4 inhibitors enhance GRE-dependent transcription in a manner similar to that described for long-

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acting β_2-adrenoceptor agonists (LABA) (Kaur et al., 2008). As roflumilast will be prescribed to patients with severe disease taking a LABA/ICS combination therapy, a primary objective of this study was to determine the effect of roflumilast alone and in combination with a LABA on glucocorticoid-induced gene expression. We submit that PDE4 inhibitors, by enhancing the transcription of glucocorticoid-inducible, anti-inflammatory genes above the maximum level that can be achieved by an ICS alone or an ICS/LABA combination therapy, could help reduce exacerbation frequency and improve lung function in patients with severe COPD.

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Materials and Methods

Generation of a Stable 2×GRE Luciferase Reporter in BEAS-2B Cells. Stable transfection was used to generate a GRE reporter cell line. The construct, pGL3.neo.TATA.2GRE, contains two copies of a consensus simple GRE site (sense strand, 5'-TGT ACA GGA TGT TCT-3') positioned upstream of a modified minimal β-globin promoter driving a luciferase gene and a separate neomycin gene to confer resistance to geneticin (see Kaur et al., 2008). BEAS-2B cells at ~70% confluence in T162 flasks were transfected with 8 µg of plasmid DNA and 20µl of lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). After 24h, geneticin (200µg/ml) was added until foci of stable transfectants appeared, which were harvested to create heterogeneous populations of cells in which the site of integration was randomized.

Culture of 2×GRE BEAS-2B Reporter Cells and Measurement of Luciferase. Cells were cultured for two days under a 5% CO_2/air atmosphere at 37^{o}C in 24-well plastic plates (Corning Life Sciences, Lowell, MA) containing DMEM/F12 (Invitrogen) supplemented with 10% FBS (Invitrogen), L-glutamine (2.5mM) and sodium bicarbonate (0.15%v/v). The cells were then cultured for a further 24h in serum-free medium (SFM) and treated with fluticasone propionate (FP) or dexamethasone in the absence and presence of PDE4 inhibitors and/or formoterol as indicated. At 6h, cells were treated with 1x reporter lysis buffer (Promega, Madison, WI) and luciferase activity was measured using a Monolight Luminometer (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Data are expressed as -fold induction of luciferase relative to unstimulated cells.

Culture of Human Primary Airway Epithelial Cells (HpAECs). Cells were obtained from both male and female donors by proteinase digestion of non-transplanted normal human lung (International Institute for the Advancement of Medicine, Edison, NJ) and seeded in 12-well plates (Corning) containing bronchial epithelial cell growth medium (BEGM; Lonza, Walkersville, MD) supplemented with penicillin (50µg/ml) and streptomycin (10µg/ml). Initially, the cells were cultured under a 5% CO_2/air atmosphere at 37^{o}C until confluent (typically 14 days; medium changed every 3 to 4 days). They were then cultured for a further 24h in supplement-free, bronchial epithelial cell basal medium (BEBM; Lonza) and processed as described below. Ethics approval for the use of human tissues has

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been granted by the Conjoint Health Research Ethics Board of the University of Calgary.

Transient Transfection of HpAECs with a 2×GRE-Reporter Construct. Plasmid DNA (500ng) containing the pGL3.neo.TATA.2GRE construct was incubated for 30min with 0.5µl of Lipofectamine 2000 (Invitrogen) in 100µl of BEBM (without supplements) at room temperature. HpAECs at ~85% confluence in 24-well plates were washed and transfected by the addition of 250µ1 of BEBM containing DNA/lipofectamine 2000 for 6h. The complex was removed and the cells were incubated with 500µl BEGM with supplements for 18h and then starved for an additional 24h in BEBM (without supplements) before beginning experiments. Cells were harvested at 6h for luciferase determination as described above.

Infection of 2×GRE BEAS-2B Reporter Cells with Ad5.CMV.PKIα. Cells were infected (MOI = 30) with an adenovirus vector (Ad5.CMV.PKIα) encoding the complete amino acid sequence of the α-isoform of cAMP-dependent protein kinase (PKA) inhibitor (PKIα) downstream of the constitutively active CMV immediate early promoter. The expression of the PKIα transgene was confirmed by western blotting. See Meja et al., (2004) for further details.

Measurement of cAMP. BEAS-2B cells at 37^{o}C were grown to confluence in 12 well plates, growth arrested for 24h in SFM and incubated for 1h with vehicle, roflumilast (2nM), formoterol (100pM) and roflumilast and formoterol in combination. The SFM was decanted and the cells lysed in HCl (0.1M). cAMP was measured in cell lystes by ELISA (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions and is expressed in units of pmol/ml.

RNA Isolation, Reverse Transcription and Real-time qPCR. Total RNA was extracted from BEAS-2B cells and HpAECs that had been treated with glucocorticoid, PDE4 inhibitor or LABA alone and in combination as indicated using RNeasy Mini Kits (Qiagen Inc., Mississauga, ON, Canada) and was reverse transcribed using a qscript cDNA synthesis kit according to the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). Real-time qPCR analysis of cDNA using the primer sequences shown in Table 1 (designed using *Primer Express*® software, Applied Biosystems Inc, Foster City, CA), encoding glucocorticoid-induced leucine zipper (GILZ;

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gene name: transforming growth factor β-stimulated clone 22, domain family member 3 [*TSC22D3*]), kinase inhibitor protein 2 of 57 kDa ($p57^{kip2}$; gene name: cyclin-dependent kinase inhibitor 1C [*CDKN1C*]), cysteine-rich secretory protein LCCL (Limulus clotting factor C, Cochlin, Lgl1) domain-containing 2 (CRISPLD2), regulator of G-protein signalling 2 (RGS2) and cluster of differentiation 200 (CD200) was performed using an ABI 7900HT instrument (Applied BioSystems) on 2.5µl of cDNA in 10µl reactions using SYBR GreenER chemistry (Invitrogen) according to the manufacturer's guidelines. Relative cDNA concentrations were determined from a cDNA standard curve that was analyzed simultaneously with the test samples. Amplification conditions were: 50°C, 2min; 95°C, 10min; followed by 40 cycles of: 95°C, 15s; 60°C, 1 min. Dissociation (melt) curves (95°C, 15s; 60°C, 20s; with ramping to 95°C over a period of 20min; 95°C, 15s) were constructed to confirm primer specificity.

Curve Fitting. Monophasic, agonist concentration-effect (E/[A]) curves were fitted by least-squares, non-linear iterative regression to the following form of the Hill equation using Prism 4 (GraphPad Software Inc, San Diego, CA):

$$E = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{(p[A]_{50} - p[A])^n}} \qquad (1)$$

where E is the effect, E_{min} and E_{max} are the lower and upper asymptote (i.e. the basal response and maximum agonist-induced response respectively), p[A] is the log molar concentration of agonist, $p[A]_{50}$ is a location parameter equal to the log molar concentration of agonist producing (E_{max}-E_{min})/2 and n is the gradient of the E/[A] curve at the $p[A]_{50}$ level.

Drugs and Analytical Reagents. Roflumilast (3-(cyclopropylmethoxy)-*N*-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide), roflumilast *N*-oxide 3-(cyclopropylmethoxy)-*N*-(3,5-dichloro-1-oxidopyridin-4-yl)-4-(difluoromethoxy) benzamide), cilomilast (4-cyano-4-[3-(cyclopentyloxy)-4-methoxyphenyl] cyclohexane carboxylic acid), tetomilast (6-[2-(3,4-diethoxyphenyl)-1,3-thiazol-4-yl]pyridine-2-carboxylic acid), apremilast (*N*-[2-[(1*S*)-1-(3-ethoxy-4-methoxyphenyl)-2-

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methylsulphonylethyl]-1,3-dioxoisoindol-4-yl]acetamide)) and AWD 12-281 were synthesised by Nycomed (Konstanz, Germany). GSK 256066 and dexamethasone were from Gilead Sciences (Seattle, WA) and Steraloids (Newport, RI) respectively. Formoterol, FP and all other drugs, chemicals and reagents were from Sigma-Aldrich (Oakville, Ontario, Canada). PDE4 inhibitors, formoterol and glucocorticoids were dissolved in DMSO and diluted to the desired working concentrations in culture medium. The final concentrations of DMSO did not exceed 0.5% (v/v).

Definitions and Statistics. In the text, the term "additivity" refers to an effect produced by two or more drugs that, when combined, is the sum of their individual components. Conversely, the term "positive cooperativity" describes a response produced by two or more drugs in combination that is greater than the sum of their individual effects.

Data points and values in the text and figure legends are presented as the mean ± s.e. mean of *N* independent determinations. Data were analyzed by using a one-way or two-way ANOVA as indicated followed, when appropriate, by Tukey's multiple comparison test. In the gene expression studies, all statistical analyses were performed on untransformed data. The null hypothesis was rejected when $P < 0.05$.

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Results

Effect of PDE4 Inhibitors on GRE-dependent Transcription in BEAS-2B Cells: A Comparison with the LABA, Formoterol. Treatment of 2×GRE BEAS-2B reporter cells with FP (0.1nM – 1μM) for 6h induced GRE-dependent transcription in a concentration-dependent manner with a $[A]_{50}$ and E_{max} of 3.1nM and 18-fold respectively (Fig. 1*A* & *B;* Table 2). In contrast, neither roflumilast (1μM) nor cilomilast (10μM) alone induced the expression of luciferase (data not shown). However, when added to 2×GRE BEAS-2B reporter cells concurrently with FP, both PDE4 inhibitors enhanced (1.6- to 1.9-fold), in a positive cooperative fashion, FP-induced transcription above that produced by the glucocorticoid alone (Fig. 1*A* & *B*). Analysis of the *E*/[A] relationship that described this effect showed that both roflumilast and cilomilast increased the magnitude of transcription (i.e. E_{max} was enhanced) without significantly affecting the potency of FP (i.e. $[A]_{50}$ was unchanged; Fig. 1*A* & *B;* Table 2). In the presence of a maximally effective concentration of FP (100nM) determined from the studies shown in figure 1*A* and *B*, cilomilast and roflumilast enhanced GRE-dependent transcription in a concentration-dependent manner with maximal effects being 2- to 2.5-fold greater than that produced by FP alone (Fig. 1*C* & *D*; Table 3).

Consistent with a previous report (Kaur et al., 2008), formoterol also enhanced FP (100nM)-induced, GRE-dependent transcription in BEAS-2B cells harbouring the same 2×GRE construct (Fig. 1*C* & *D;* Table 3). However, the maximum fold induction produced by formoterol was significantly greater than that produced by either cilomilast or roflumilast (Fig. 1*A*-*D;* Tables 2 & 3) in the absence of any change in the potency of FP (Fig. 1*A* & *B;* Table 2).

The interaction between FP and roflumilast and FP and cilomilast was not peculiar to these drug combinations. Indeed, six other PDE4 inhibitors of varied structural classes (AWD 12-281, apremilast, GSK 256066, rolipram, roflumilast *N*-oxide, tetomilast) were compared using dexamethasone (1μM) as a representative glucocorticoid. In every case, GRE-dependent transcription was significantly augmented (1.8- to 2.1-fold; Fig. 2).

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Effect of Cilomilast and Roflumilast on the Kinetics of GRE-dependent Transcription in BEAS-2B Cells. Treatment of 2×GRE BEAS-2B reporter cells with FP (100nM) induced the luciferase gene in a time-dependent manner (Fig. 3*A* & *B*). Transcription reached a maximum at the 6h time-point and this level of luciferase activity then gradually declined over the next 18h. In the presence of roflumilast (1μM) or cilomilast (10μM), which were inactive alone on the reporter, the ability of FP to promote GRE-dependent transcription was augmented at all time-points examined (Fig. 3*A* & *B*). The greatest effects were seen at 4-6h and this additional level of luciferase induction was largely preserved over the time-course of the experiment although the absolute luciferase activity waned (Fig. 3*A* & *B*).

Effect of Selective Inhibitors of other cAMP PDEs on GRE-dependent Transcription in BEAS-2B Cells. BEAS-2B cells and HpAECs express multiple cAMP PDEs including PDE2, PDE3, PDE4 and PDE7 (Dent et al., 1998, Fuhrmann et al., 1999, Smith et al., 2003). Figure 4 shows the effect on GRE-dependent transcription of inhibitors of these PDEs at the highest concentrations where isoenzyme selectivity is preserved. None of the PDE inhibitors alone activated the 2×GRE BEAS-2B reporter whereas dexamethasone (1μM) increased luciferase activity by 19.4-fold. Selective inhibitors of PDE2 (Bay 60-7550; 500nM) and PDE3 (siguazodan; 10μM) each added concurrently with dexamethasone had no further effect on GRE-dependent transcription (Fig. 4*A*). Indeed, siguazodan (10μM) did not significantly modify the dexamethasone *E*/[A] relationship in terms of either its $p[A]_{50}$ or E_{max} (Fig. 4*B*). In contrast, BRL 50481 (100μM), an inhibitor of PDE7 (Smith et al., 2004), and rolipram (10μM), an archetypical PDE4 inhibitor significantly enhanced dexamethasone-induced luciferase activity from 19.4-fold to 25- and 45-fold respectively (Fig. 4*A*). Moreover, when added concurrently with dexamethasone to 2×GRE BEAS-2B reporter cells, rolipram and BRL 50481 increased transcription at 6h to 53.3-fold. All other cAMP PDE inhibitor combinations that were examined failed to enhance dexamethasone-induced GRE-dependent transcription to a greater extent than rolipram alone (Fig. 4*A*).

Effect of PKIα on the Enhancement by Roflumilast, Roflumilast *N*-Oxide and Cilomilast of GRE-dependent Transcription in BEAS-2B Cells. To examine a possible role for PKA in the

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enhancement of GRE-dependent transcription by PDE4 inhibitors, a highly selective, endogenous inhibitor, PKIα, was used that was delivered to BEAS-2B cells by an adenovirus vector, Ad5.CMV.PKIα (see Meja et al., 2004 and references therein). In uninfected cells, PKIα was not detected by western blotting in any experiment. However, 48h after infection of BEAS-2B cells with Ad5.CMV.PKIα (MOI = 30), a single protein was labelled by the anti-PKIα antibody that migrated as a 12 kDa band on SDS polyacrylamide gels (Fig 5*A*). As shown in figure 5*B*, the concentration-dependent enhancement by cilomilast of FP (100nM)-induced GRE-dependent transcription was abolished in cells expressing the PKIα transgene. In contrast, cells infected with a null virus, Ad5.CMV.Null, responded to cilomilast in a manner that was not significantly different from uninfected cells (Fig. 5*B*). Identical results were obtained with roflumilast (1μM) and its active metabolite, roflumilast *N*-oxide (1μM; Fig. 5*C*).

Effect of Roflumilast on the Ability of Formoterol to Augment GRE-dependent Transcription in BEAS-2B Cells. We have reported previously that the LABA, formoterol, augments GRE-dependent transcription in 2×GRE BEAS-2B reporter cells (Giembycz et al., 2008, Kaur et al., 2008). Figure 6 shows the results of studies designed to assess whether this effect of formoterol could be potentiated by the PDE4 inhibitor, roflumilast. FP (100nM), alone, increased luciferase activity by ~17-fold (Fig. 6*A*) and this effect was augmented in a concentration-dependent manner by formoterol ($p[A]_{50}$ = -10.4 ± 0.02; E_{max} = 46-fold; Fig. 6*A*). Roflumilast, which by itself, enhanced GRE-dependent transcription, significantly potentiated the effect of formoterol in a concentration-dependent manner ($p[A]_{50}$s = -10.69 ± 0.03, -11.03 ± 0.02 and -11.2 ± 0.02 in the presence of 1nM, 10nM and 100nM roflumilast respectively) although the maximum luciferase activity was unchanged (Fig. 6*A*). Re-plotting these data (Fig. 6*B*), showed that roflumilast at concentrations of 10nM and higher significantly potentiated the effect of formoterol (10pM). On cAMP a similar effect was observed. Thus, roflumilast (2nM), which was inactive by itself, significantly augmented formoterol-induced cAMP accumulation (from 75.2 ± 11.3 to 118.6 ± 5.3 pmol/ml; Fig. 6*C*) when measured at a time (1h) that preceded the peak in GRE-dependent transcription (Fig. 3).

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Effect of Roflumilast and Cilomilast on FP-induced GRE-dependent Transcription in HpAECs. Additional studies were performed to confirm that PDE4 inhibitors and a LABA can augment GRE-dependent transcription in HpAECs transiently transfected with the same 2×GRE reporter construct. As shown in figure 7, FP (100nM) doubled luciferase expression when compared to unstimulated cells and this effect was enhanced if formoterol (1nM) was added concurrently with the glucocorticoid. Significantly, formoterol (100pM) and low concentrations of roflumilast (2nM) or cilomilast (20nM) that, by themselves, did not significantly enhance FP-induced transcription, interacted in combination to produce an effect that was equal to that evoked by a maximally-effective concentration of formoterol (1nM).

Effect of Roflumilast and Fomoterol alone and in combination on the Expression of Glucocorticoid-inducible Genes. The data presented in the preceding sections demonstrate that a PDE4 inhibitor and/or formoterol, alone and in combination, enhanced glucocorticoid-induced transcription from a conventional, simple 2×GRE reporter. To determine whether these findings translate to the enhancement of real genes, we used data derived from two microarrays in which dexamethasone- and indacaterol-inducible mRNA transcripts were identified in pulmonary type II A549 cells and BEAS-2B cells respectively. As briefly outlined in table 4, there is literature precedent that the expression of several of these genes including *GILZ*, *RGS2*, *p57*^{kip2}, *CRISPLD2* and *CD200* could suppress indices of inflammation and so reduce exacerbations, protect against bronchoconstriction and limit airway remodelling (Eddleston et al., 2007, Holden et al., 2011, Samuelsson et al., 1999, Snelgrove et al., 2008, Wang et al., 2009). Accordingly, these genes were selected to determine if they are regulated in HpAECs and BEAS-2B cells in a manner similar to the 2×GRE reporter.

HpAECs. Initially, a small proof of concept experiment was performed to assess the ability of a maximally-effective concentration of roflumilast (1μM; determined from the data presented in figure 1*D*) to augment FP-induced *GILZ* expression in HpAECs. To ensure the detection of any additive or positive cooperative interactions between these stimuli, quantitative PCR was performed using cDNA prepared from cells treated over an 18h time-frame. As shown in figure 8, FP (100nM) increased

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GILZ expression in a time-dependent manner by a mechanism that was enhanced by roflumilast (from 1.1- to 3.3-fold, 2.9- to 8.9-fold, 2.5- to 13.5-fold and 16.9- to 43.6-fold at 1h, 2h, 6h and 18h respectively). Roflumilast (1μM), alone, was inactive. Based on these results, all further gene expression analyses in HpAECs were performed at 2h and 6h and were expanded to include *RGS2* $p57^{kip2}$, *CD200* and *CRISPLD2*. In BEAS-2B cells, mRNA transcripts were measured 6h after treatments as we have reported previously (Kaur et al., 2008, Wilson et al., 2009). Finally, in both cell types, the concentration of roflumilast was reduced to 2nM. This equates to the amount of free (unbound) roflumilast *N*-oxide (the primary, active metabolite), in plasma at steady state after administration of the recommended dose (500μg o.d.) (Bethke et al., 2007), which inhibits PDE4 subtypes with potencies similar to the parent compound (Hatzelmann et al., 2010).

Treatment of HpAECs with FP (100nM) induced *RGS2* at 2h and 6h by 9.2- and 4.6-fold respectively relative to time-matched, untreated cells (Fig. 9*A* & *B*). Neither formoterol nor roflumilast induced *RGS2* at either time-point. However, at 2h formoterol significantly enhanced the effect of FP (to 14.2-fold) although no further increase was seen if roflumilast was combined with formoterol. At 6h, formoterol failed to enhance FP-induced *RGS2* expression whereas a significant augmentation was produced when FP, formoterol and roflumilast were used in combination (to 8.7-fold; Fig. 9*B*).

At 2h and 6h, FP, formoterol and roflumilast failed to induce $p57^{kip2}$ (Fig. 9*C* & *D*). However, in combination, FP and formoterol increased $p57^{kip2}$ expression by 3.1- and 6.2-fold at 2h and 6h respectively. A combination of formoterol and roflumilast enhanced FP-induced $p57^{kip2}$ expression at 2h to a significantly greater degree (to 4.4-fold) than did formoterol alone although this interaction was lost at 6h.

Formoterol and roflumilast alone significantly enhanced the ability of FP to induce *GILZ* at 2h (from 6.5-fold to 19.5-fold and 12.3-fold respectively) although no further increase was seen if formoterol was combined with roflumilast (Fig. 9*E*). At 6h, a similar profile of activity was found with the exception that roflumilast alone did not augment FP-induced *GILZ* expression (Fig. 9*F*).

None of the interventions significantly induced *CD200* in HpAECs at either time point (Fig. 9*G* & *H*)

Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
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or of *CRISPLD2* at 2h (Fig. 9*I*). However, at 6h, FP and roflumilast in combination increased the expression of *CRISPLD2* that was significantly greater than in time-matched, unstimulated cells (Fig. 9*J*).

BEAS-2B Cells. Treatment of BEAS-2B cells with FP (100nM) or formoterol (100pM) produced a 3.1- and 2.6-fold induction of *RGS2* respectively at 6h, whereas roflumilast (2nM) was ineffective (Fig. 10*A*). Formoterol, but not roflumilast, interacted in a positive cooperative manner with FP and augmented *RGS2* expression levels by 21-fold relative to time-matched, untreated cells. Similarly, treatment of cells concurrently with FP, formoterol and roflumilast further enhanced *RGS2* expression to a level above that produced by the combination of FP and formoterol (30-fold; Fig. 10*A*). Roflumilast and formoterol in combination also induced *RGS2* expression (3.8-fold) but this was not significantly greater than the effect produced by formoterol (Fig. 10*A*).

The expression of $p57^{kip2}$, *CD200* and *CRISPLD2* following exposure of BEAS-2B cells to FP, roflumilast and formoterol individually and in their various combinations, was similar to that found for *RGS2*. Thus, FP (100nM) or formoterol (100pM) produced an 8.3- and 1.9-fold induction of $p57^{kip2}$ respectively at 6h whereas roflumilast (2nM) was inactive (1.2-fold; Fig. 10*B*). Formoterol, but not roflumilast, interacted in a positive cooperative manner with FP and augmented $p57^{kip2}$ mRNA levels by 23-fold relative to time-matched, untreated cells. Similarly, treatment of cells concurrently with FP, formoterol and roflumilast further enhanced $p57^{kip2}$ expression to a level that was significantly higher than the sum of the effects produced by FP, formoterol and roflumilast alone, and the combination of FP and formoterol (29-fold; Fig. 10*B*). Roflumilast and formoterol together also induced $p57^{kip2}$ expression although this effect was modest (2.5-fold; Fig. 10*B*).

mRNA levels for CD200 after a 6h exposure of BEAS-2B cells to FP, formoterol and roflumilast were 2.2-, 4.1- and 1.2-fold higher respectively relative to time-matched, untreated cells (Fig. 10*C*). Formoterol and FP together acted in an approximately additive fashion (6.8-fold induction) whereas no such interaction on *CD200* expression was seen in cells exposed concurrently to FP and roflumilast (Fig. 10*C*). However, in combination, FP, formoterol and roflumilast enhanced *CD200*

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expression to a level that was significantly higher (10.2-fold) than that the sum of the effects produced by FP, formoterol and roflumilast alone, and the combination of FP and formoterol (Fig. 10*C*).

FP (100nM) or formoterol (100pM) produced a 6.5- and 8.1-fold induction of *CRISPLD2* respectively whereas roflumilast (2nM) was inactive (1.3-fold; Fig. 10*D*). Formoterol, but not roflumilast, interacted with FP in a positive cooperative manner augmenting *CRISPLD2* mRNA levels by 25.5-fold relative to time-matched, untreated cells. Exposure of cells concurrently to FP, formoterol and roflumilast further enhanced *CRISPLD2* expression (by 35-fold) to a level that was higher than the sum of the effects produced by FP, formoterol and roflumilast alone and the combination of FP and formoterol (Fig. 10*D*).

Treatment of BEAS-2B cells with FP (100nM) produced a 49-fold induction of *GILZ* at 6h whereas formoterol (100pM) and roflumilast (2nM), individually and in combination, were inactive (Fig. 10*E*). Formoterol, but not roflumilast, augmented FP-induced *GILZ* expression to 64.5-fold relative to time-matched, untreated cells although this was not statistically significant. Similarly, treatment of cells concurrently with formoterol and roflumilast enhanced FP-induced *GILZ* expression to a similar degree (63.5-fold) as formoterol but, again, this effect was not significantly greater than that produced by FP alone or the combination of FP and formoterol (Fig. 10*E*).

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Discussion

The major finding of this study was that a panel of structurally dissimilar PDE4 inhibitors including roflumilast and its primary metabolite, roflumilast *N*-oxide, augmented GRE-dependent transcription in BEAS-2B cells and HpAECs in a manner similar to LABAs (Giembycz et al., 2008, Kaur et al., 2008). Moreover, positive cooperative effects were often observed when a PDE4 inhibitor and a LABA were combined. These interactions were produced in a simple, 2×GRE luciferase reporter *and*, more importantly, in epithelial cells in which the expression of a selection of glucocorticoid-inducible genes was determined. Indeed, formoterol and roflumilast in combination enhanced FP-induced gene expression to a significantly greater degree than either the LABA or PDE4 inhibitor alone. As outlined in the Introduction, *trans*activation is now believed to constitute a major mechanism of glucocorticoid action, which likely operates in tandem with the more established process of *trans*repression. Indeed, the expression of the anti-inflammatory gene, *GILZ*, is significantly up-regulated in bronchial biopsies harvested from human asthmatic subjects given inhaled budesonide (Kelly et al., 2012). On this basis, we propose that roflumilast in combination with an ICS may be more effective in reducing exacerbation frequency and improving lung function in patients with severe COPD relative to either drug alone due to their ability, at least in part, to interact in a positive cooperative manner on the expression of a variety of glucocorticoid-inducible, anti-inflammatory genes. Moreover, in the context of COPD treatment guidelines, enhanced gene *trans*activation could provide a mechanistic rationale for "adding-on" a PDE4 inhibitor to patients with severe COPD taking an ICS/LABA combination therapy that still suffer frequent exacerbations. In this respect, in 2011, the **REACT (R**oflumilast in the prevention of COPD **E**xacerbations while taking **A**ppropriate **C**ombination **T**reatment) study was initiated to compare the effects of roflumilast and placebo on exacerbation rates in patients who are treated simultaneously with a fixed combination of a LABA and an ICS, with or without a long-acting muscarinic receptor antagonist (Calverley et al., 2012). The results of this trial are awaited with interest.

Effect of PDE Inhibitors on GRE-dependent Transcription. In 2×GRE BEAS-2B reporter cells, PDE4 inhibitors augmented dexamethasone- and FP-induced luciferase activity in a time- and

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concentration-dependent manner. Inspection of the *E*/[A] curves showed that PDE4 inhibitors increased the ability of FP to *trans*activate the reporter without affecting its potency. This was a positive cooperative interaction because the PDE4 inhibitors alone were inactive. Of potential clinical significance was the additional observation that roflumilast potentiated the ability of formoterol to promote GRE-dependent transcription, which was described by a sinistral displacement of formoterol *E*/[A] curves. Consistent effects were obtained with HpAECs transiently transfected with the same GRE reporter indicating that these effects were not peculiar to BEAS-2B cells.

Augmentation of GRE-dependent transcription was apparently restricted to inhibitors of PDE4. However, a high concentration (100μM) of the PDE7 inhibitor, BRL 50481 (Smith et al., 2003), also increased reporter activity and interacted additively with rolipram. Whether these were genuine additive effects due to inhibition of both PDE4 and PDE7 family members or an action of BRL 50481 on residual PDE4 activity that was not inhibited by 10μM rolipram is unclear.

Effect of PDE4 Inhibitors on Gene Expression. The results of the reporter experiments prompted us to determine if "real" glucocorticoid-inducible genes are regulated similarly. Microarray profiling of A549 and BEAS-2B cells identified several candidate genes that were induced by dexamethasone and/or the LABA, indacaterol, including *RGS2*, *GILZ*, *p57*^{kip2}, *CD200* and *CRISPLD2*. Although up-regulation of these genes may, collectively, have clinical benefit in COPD (Table 4), they were selected, primarily, to provide proof-of-concept.

In HpAECs, a high concentration of roflumilast (1μM), sufficient to abolish PDE4 activity in intact cells (Hatzelmann et al., 2010), augmented FP-induced *GILZ* expression in a time-dependent manner. In contrast, a lower concentration (2nM) of roflumilast, comparable to the amount of free, unbound roflumilast *N*-oxide (which is equi-potent) in plasma at steady state after administration of the recommended dose (Bethke et al., 2007), was inactive on the six genes studied. However, roflumilast and formoterol in combination enhanced the expression of several FP-inducible genes to levels that were significantly greater than those produced by formoterol alone. Although differences were apparent between BEAS-2B cells and HpAECs with regard to the genes affected, the magnitude of

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gene induction and their sensitivity to formoterol and roflumilast, the data gathered from the two cell types were conceptually consistent. The reason(s) why these two cells types do not respond equivalently to glucocorticoids and/or cAMP-elevating drugs is unclear, but our experience is that the composition of culture media, passage number and reagents used for the purification of primary epithelial cells are contributing factors.

The inactivity of a low concentration of roflumilast may suggest that *in vitro* a degree of tonic, cAMP "drive" is required for an impact on FP-induced gene expression to occur. This may be particularly relevant in cells such as airway epithelia that do not make appreciable quantities of cAMP-elevating autacoids such as PGE_2 (*cf.* airway myocytes; Lazzeri et al., 2001). However, *in vivo*, cAMP-generating ligands will be released from a variety of cell types as well as through the therapeutic consumption of β_2-adrenoceptor agonists.

Mechanism of action of PDE4 inhibitors. cAMP measurements and studies with the PKIα expression vector indicated that roflumilast, roflumilast *N*-oxide and cilomilast augmented GRE-dependent transcription by activating the canonical cAMP/PKA signalling cascade. Although the downstream molecular targets of PKA are unknown, at least two possible mechanisms could explain this phenomenon. First, PDE4 inhibitors may augment the translocation of GRs from the cytosol to the nucleus. While evidence is available to support this proposal (Miller et al., 2002), it necessarily implies that the transcription of *all* glucocorticoid-inducible genes would be enhanced by a cAMP-elevating drug, which does not seem to be the case (Giembycz et al., 2008, Kaur et al., 2008, Newton et al., 2010). Thus, to account for the enhancement of transcription of only a specific subset of glucocorticoid-inducible genes, it is necessary to consider alternative explanations. One possibility is PKA-mediated phosphorylation of components of the transcriptional apparatus. For example, the human GR features multiple phosphorylation sites of which S^{211} is phosphorylated by PKA (Galliher-Beckley and Cidlowski, 2009, Miller et al., 2007). It is possible that phospho-GR would be better able to drive the transcription of some, but not all, glucocorticoid-inducible genes. Alternatively, PKA may target substrates downstream of the GR that regulate the activity and/or recruitment of specific co-factors (Moyer et al., 1993). While such regulation of glucocorticoid-inducible genes remains

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undefined, phosphorylation could confer promoter-specificity and explain why the expression of some glucocorticoid-inducible genes is augmented in a positive cooperative fashion by cAMP-elevating agents, while others are not.

Clinical Relevance. The concentration of free, unbound roflumilast *N*-oxide in the plasma of COPD patients at steady state (~2nM; Bethke et al., 2007) after administration of the recommended dose of the parent compound, roflumilast, was at the threshold for enhancing GRE-dependent transcription in epithelial cells. One interpretation of these data is that this effect of roflumilast has limited clinical significance. However, many cells in the lung generate a variety of cAMP-elevating autacoids (e.g. PGE_2, adenosine) with which a low dose of a PDE4 inhibitor could interact in a positive cooperative fashion in a way similar to that shown for the induction of *RGS2* and $p57^{kip2}$. Credibility for this idea derives from the fact that the concentrations of PGE_2 and ATP (which is readily dephosphorylated into adenosine) are elevated in the exhaled breath condensate and in bronchoalveolar lavage fluid respectively of patients with COPD when compared to control subjects (Lommatzsch et al., 2010, Montuschi et al., 2003). Moreover, roflumilast is recommended for patients with severe disease (GOLD stages 3/4) that will already be taking a LABA/ICS combination therapy and a SABA, as a rescue medication.

Conclusions. The beneficial effects of roflumilast in COPD are suggested to be due to the suppression of inflammation (Hatzelmann et al., 2010). The results presented herein provide a novel mechanism to account for this anti-inflammatory effect, especially in patients with severe disease taking ICS/LABA combination therapy. We submit that an ICS, a LABA and a PDE4 inhibitor in their various combinations work in several distinct, but mutually cooperative, ways (Fig. 11). Foremost, PDE4 inhibitors, particularly in the presence of a LABA, enhance the expression of some glucocorticoid-induced genes to levels that cannot be achieved by a glucocorticoid alone. A notable example is $p57^{kip2}$, which was markedly induced by FP, formoterol and roflumilast in combination, but largely unaffected by these drugs individually. Some of these same ICS-inducible genes may also be up-regulated by a LABA (e.g. *CD200*). In addition (although not examined here), a LABA and/or PDE4 inhibitor may up-regulate cAMP-inducible, anti-inflammatory genes that are *insensitive* to

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glucocorticoids, which could contribute to the therapeutic activity of roflumilast reported in COPD patients not taking ICS (Calverley et al., 2009). In concluding, it is appropriate to acknowledge that although LABAs and PDE4 inhibitors alone can *induce* a variety of pro-inflammatory genes, this unwanted effect could be mitigated by an ICS and may not be overly problematic (Holden et al., 2010; Hertz et al., 2009). Thus, in severe COPD, the clinical efficacy of an ICS, a LABA and PDE4 inhibitor in combination may be attributable, in part, to their collective actions on gene transcription (Giembycz and Newton, 2011).

Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
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Acknowledgement

The authors thank Dr. Hermann Tenor for providing constructive comments on this manuscript.

Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Giembycz, Newton

Conducted experiments: Joshi, Moodley, Rider, Sharma, Wilson, Yan

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Wrote or contributed to the writing of the manuscript: Giembycz, Newton

Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
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Footnotes

This study was supported by the Canadian Institutes for Health Research [CIHR; MOP 93742], the Lung Association, Alberta and North West Territories and an unrestricted research grant from Nycomed Inc, USA. The authors state no conflict of interest.

Molecular Pharmacology Fast Forward. Published on February 6, 2013 as DOI: 10.1124/mol.112.083493
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Figure Legends

Fig. 1. Effect of cilomilast and roflumilast on luciferase activity in 2×GRE BEAS-2B reporter cells. In panels *A* and *B* cells, were treated with fluticasone propionate (FP; 10pM to 1μM) in the absence and presence of cilomilast (10μM), roflumilast (1μM) or formoterol (1nM), as indicated. In panels *C* and *D*, *E*/[A] curves were constructed to cilomilast (1nM to 30μM), roflumilast (100pM to 3μM) and formoterol (1pM to 3nM) in the presence of a fixed concentration of FP (100nM). At 6h cells were harvested for the determination of luciferase activity. Data points and bars represent the mean ± s.e. mean of *N* independent determinations. The dashed lines in panels *C* and *D* define the effect of FP alone. See tables 2 and 3 for further details.

Fig. 2. Effect of structurally-dissimilar PDE4 inhibitors on luciferase activity in 2×GRE BEAS-2B reporter cells. In panel *A*, cells were treated with dexamethasone (Dex; 1μM) in the absence and presence of rolipram (10μM), AWD 12-821 (10μM), tetomilast (30μM), roflumilast (1μM) or roflumilast *N*-oxide (1μM). Panel *B* shows a separate experiment in which cells were treated with dexamethasone (1μM) in the absence and presence of apremilast (5μM), cilomilast (10μM) and GSK 256066 (10nM). At 6h cells were harvested for the determination of luciferase activity. Bars represent the mean ± s.e. mean of *N* independent determinations as indicated in each panel. The dashed lines define the effect of dexamethasone alone.

* and ** $P < 0.05$, significant enhancement in luciferase activity relative to unstimulated cells (NS) and dexamethasone respectively. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test.

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Fig. 3. Kinetics of GRE-dependent transcription in 2×GRE BEAS-2B reporter cells. Confluent cells were treated with fluticasone propionate (FP; 100nM), cilomilast (10μM), roflumilast (1μM) or a combination of FP with either PDE4 inhibitor. Cells were then incubated for 1 to 24h and harvested for luciferase activity. Panels *A* and *B* refer to the FP/cilomilast and FP/roflumilast combinations. Data points represent the mean ± s.e. mean of *N* independent determinations.

* $P < 0.05$, significant enhancement of transcription relative to time-matched, unstimulated cells.

+ $P < 0.05$, significant enhancement of transcription relative to time-matched, FP-treated cells.

Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test.

Fig. 4. Effect of isoenzyme-selective PDE inhibitors on luciferase activity in 2×GRE BEAS-2B reporter cells. In panel *A*, cells were treated with dexamethasone (Dex; 1μM) in the absence and presence of Bay 60-7550 (Bay: 500nM), siguazodan (Sig; 10μM), rolipram (Roli; 10μM), BRL 50481 (BRL; 100μM) or the following combinations: Bay 60-7550 and siguazodan, Bay 60-7550 and rolipram, Bay 60-7550 and BRL 50481, siguazodan and rolipram, siguazodan and BRL 50481, and rolipram and BRL 50481. In panel *B*, cells were treated with dexamethasone (100pM to 1μM) in the absence and presence of the PDE3 inhibitor, siguazodan (10μM). At 6h cells were harvested for the determination of luciferase activity. Bars and data points represent the mean ± s.e. mean of *N* independent determinations shown in parentheses. The dashed line in panel *A* defines the effect of dexamethasone alone. The numerals at the base of each bar in panel *A* refer to the PDE family targeted by the inhibitor(s).

* $P < 0.05$, significant enhancement in luciferase activity relative to dexamethasone-treated cells. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test. NS: Unstimulated cells.

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Fig. 5. Effect of PKIα on the enhancement of GRE-dependent transcription by cilomilast, roflumilast and roflumilast *N*-oxide. 2×GRE BEAS-2B reporter cells were infected with Ad5.CMV.PKIα, Ad5.CMV.Null (both MOI = 30) or left untreated. Cells were subsequently subjected to western analysis using a polyclonal antibody directed against PKIα or β-actin (panel *A*) or were stimulated with cilomilast (10 μM; panel *B*) or roflumilast (1μM) and roflumilast *N*-oxide (1μM; panel *C*) in the presence of fluticasone propionate (FP; 100nM). After 6h cells were harvested for the determination of luciferase activity. Bars represent the effect of FP in cells left untreated (filled) or infected with Ad5.CMV.Null (open) or Ad5.CMV.PKIα (hatched). Data points and bars represent the mean ± s.e. mean of *N* independent determinations.

* $P < 0.05$, significant inhibition by PKIα over-expression of the enhancement of GRE-dependent transcription by roflumilast and roflumilast *N*-oxide relative to cells infected with Ad5.CMV.Null. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test.

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Fig. 6. Synergistic interaction between FP, roflumilast and formoterol on GRE-dependent transcription and cAMP levels in 2×GRE BEAS-2B reporter cells. In panel *A*, *E*/[A] curves were constructed to formoterol in cells that were treated concurrently with FP (100nM) alone and in the presence of roflumilast (1nM, 10nM and 100nM as indicated). In panel *B*, the data have been re-plotted to illustrate the additional effect that roflumilast produced on GRE-dependent transcription in cells treated with formoterol and FP, in combination at the concentrations indicated. At 6h, cells were harvested for the determination of luciferase activity. In panel *C*, cells were exposed for 1h to roflumilast (Rof; 2nM), formoterol (Form; 100pM), a combination of roflumilast and formoterol (Rof + Form) or were left untreated and cAMP was measured by ELISA. In each panel, bars and data points represent the mean ± s.e. mean of *N* independent determinations.

* $P < 0.05$, significant enhancement of transcription by roflumilast over the effect of FP (100nM) and formoterol (10pM) in combination. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test.

** and *** $P < 0.05$, significant increase in cAMP content relative to unstimulated cells (NS) and formoterol-stimulated cells respectively. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test. NSD; not significantly different.

Fig. 7. Effect of roflumilast, cilomilast and formoterol on FP-induced GRE-dependent transcription in HpAECs transiently transfected with a 2×GRE reporter. Cells were treated with FP (100nM) in the absence and presence of formoterol (100pM or 1nM), roflumilast (2nM or 1μM) or cilomilast (20nM or 10μM) alone or in the combinations indicated. At 6h cells were harvested for the determination of luciferase activity. Bars represent the mean ± s.e. mean of *N* independent determinations. The dashed line defines the effect of FP alone.

* and ** $P < 0.05$, significant enhancement of luciferase activity relative to no stimulus (NS) and FP respectively. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. NS: Unstimulated cells.

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Fig. 8. Roflumilast augments the induction of *GILZ* in HpAECs by FP. Cells were treated with FP (100nM) or roflumilast (1μM) alone and in combinations or left untreated (NS) for 1h, 2h, 6h and 18h (panels *A* to *D* respectively). cDNA was prepared and real-time RT-PCR was performed for *GILZ* and normalized to *GAPDH*. Data are expressed as -fold stimulation relative to *GAPDH* and are plotted as means ± s.e. mean of *N* independent observations. The dashed lines define the effect of FP alone.

* $P < 0.05$, significant enhancement of *GILZ* expression relative to FP-treated cells. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Fig. 9. Effect of roflumilast and formoterol, alone and in combination on the expression of FP-inducible genes in HpAECs. Cells were treated with FP (100nM), formoterol (100pM) or roflumilast (2nM) alone and in combination or left untreated (NS) as indicated. After 2h and 6h cDNA was prepared and real-time RT-PCR was performed for *RGS2* (panels *A* & *B*), $p57^{kip2}$ (panel *C* & *D*), *GILZ* (panels *E* & *F*), CD200 (panel *G* & *H*) and CRISPLD2 (panel *I* & *J*). Data are expressed as -fold stimulation relative to *GAPDH* and are plotted as means ± s.e. mean of *N* independent observations. The dashed lines define the effect of FP alone.

* $P < 0.05$, significant enhancement of transcription relative to FP alone.

+ $P < 0.05$, significant enhancement of transcription relative to FP + formoterol.

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

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Fig. 10. Effect of roflumilast and formoterol, alone and in combination, on the expression of FP-inducible genes in BEAS-2B cells. Cells were treated with FP (100nM), formoterol (100pM) or roflumilast (2nM) alone and in the combinations or left untreated (NS) as indicated. After 6h cDNA was prepared and real-time RT-PCR was performed for *RGS2* (panel *A*), *p57*^{kip2} (panel *B*), *CD200* (panel *C*), *CRISPLD2* (panel *D*) and *GILZ* (panel *E*). Data are expressed as -fold stimulation relative to *GAPDH* and are plotted as means ± s.e. mean of *N* independent observations. The dashed lines define the effect of FP alone.

* $P < 0.05$, significant enhancement of transcription relative to FP alone.

+ $P < 0.05$, significant enhancement of transcription relative to FP + formoterol.

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Fig. 11. Enhancement of glucocorticoid-dependent gene expression by cAMP-elevating agents. Glucocorticoids, such as fluticasone propionate (FP), enter the cytoplasm and bind to the glucocorticoid receptor (GR). The liganded GR then translocates to the nucleus, dimerises and interacts with glucocorticoid response elements (GRE) to promote the transcription of genes that encode proteins with anti-inflammatory activity. Agonism of the β_2-adrenoceptor (β_2R) with the LABA, formoterol, or inhibition of phosphodiesterase 4 (PDE4) with roflumilast enhances anti-inflammatory gene expression by at least two mechanisms that involve, minimally, activation of the canonical cAMP/PKA signaling cascade. Studies performed using luciferase reporters indicate that GRE-dependent transcription is, in some way, augmented by a mechanism that may involve the direct or indirect phosphorylation of GR and/or of one or more obligatory transcriptional co-activators (CoA). Activation of PKA may also up-regulate genes concurrently with, but independently of, the GR, by activating additional transcription factors including cAMP-response element binding protein (CREB) or activating transcription factor 1 (ATF-1). These transcription factors may promote gene expression by binding to cAMP response elements (CRE) that are located in some of the same genes that are up-regulated by GR and/or distinct genes that are not glucocorticoid responsive. Thus, the net effect of combining a glucocorticoid with a LABA and PDE4 inhibitor is to up-regulate anti-inflammatory gene expression to a level that cannot be achieved by any of the drugs alone.

TABLE 1. Primer pairs for real-time qPCR.

Gene	*Oligonucleotide*	*Accession Number(s)*
p57^{kip2} (*CDKN1C*)		NM_000076.2, NM_001122630.1, NM_001122631.1
Forward	5′-CGG CGA TCA AGA AGC TGT C-3′	
Reverse	5′-GGC TCT AAA TTG GCT CAC CG-3′	
GILZ (*TSC22D3*)		NM_001015881.1, NM_198057.2, NM_004089.3
Forward	5′-GGC CAT AGA CAA CAA GAT CG-3′	
Reverse	5′-ACT TAC ACC GCA GAA CCA CCA-3′	
RGS2		NM_002923
Forward	5′-CCT CAA AAG CAA GGA AAA TAT ATA CTG A-3′	
Reverse	5′-AGT TGT AAA GCA GCC ACT TGT AGC T-3′	
CD200		NM_005944.5, NM_001004196.2
Forward	5′-GGA CTG TGA CCG ACT TTA AGC AA-3′	
Reverse	5′-AGC AAT AGC GGA ACT GAA AAC C-3′	
CRISPLD2		NM_031476.3
Forward	5′-CAA ACC TTC CAG CTC ATT CAT G-3′	
Reverse	5′-GGT CGT GTA GCA GTC CAA ATC C-3′	
GAPDH		NM_002046.4, NM_001256799.1
Forward	5′-TTC ACC ACC ATG GAG AAG GC-3′	
Reverse	5′-AGG AGG CAT TGC TGA TGA TCT-3′	

Forward and reverse primers for each gene are listed and were designed to amplify all variants where multiple forms exist. Common gene names are shown and official HUGO gene symbols, if different, are given in brackets.

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TABLE 2. Effect of a fixed concentration of cilomilast, roflumilast or formoterol on the *E*/[A] relationship that describes FP-induced, GRE-dependent transcription in 2×GRE BEAS-2B reporter cells.

Treatment	***N***	***p[A]$_{50}$ (M) of FP***	***E_{max} of FP (fold)***	***Enhancement of Maximum FP-induced Response (fold)***
FP	16	-8.51 ± 0.09	18.0 ± 0.9	1
+ Cilomilast (10μM)	9	-8.35 ± 0.07	34.9 ± 1.9*	1.9
+ Roflumilast (1μM)	12	-8.29 ± 0.10	28.6 ± 1.1*	1.6
+ Formoterol (1nM)	12	-8.37 ± 0.03	41.4 ± 1.2*	2.3

$^{*}P < 0.05$, E_{max} significantly different from that produced by FP alone. Data are derived from the graphs in figure 1*A* & *B*. and were analysed by one-way ANOVA followed by Tukey's multiple comparison test.

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TABLE 3. Concentration-dependence of the enhancement of FP-induced, GRE-dependent transcription in 2×GRE BEAS-2B reporter cells by cilomilast, roflumilast and formoterol.

Treatment	*N*	*p[A]$_{50}$ (M)*	*Induction (fold)*	*Enhancement of FP-induced Response (fold)*
FP (100nM)	8	-	11.8 ± 0.7	1.0
+ Cilomilast	8	-5.48 ± 0.98	23.7 ± 3.3*	2.0
+ Formoterol	8	-10.41 ± 0.08	32.2 ± 1.3*,†	2.7
FP (100nM)	7	-	10.1 ± 1.0	1.0
+ Roflumilast	7	-7.21 ± 0.30	20.7 ± 2.8*	2.0
+ Formoterol	7	-10.45 ± 0.05	31.7 ± 0.8*,†	3.1

$^{*}P < 0.05$, E_{max} of FP response significantly augmented.

$^{\dagger}P < 0.05$, E_{max} of formoterol response significantly greater than E_{max} of cilomilast and roflumilast responses. Data are derived from the graphs in figure 1*C* & *D*. and were analysed by one-way ANOVA followed by Tukey's multiple comparison test.

TABLE 4. Glucocorticoid- and cAMP-inducible genes with potential therapeutic activity in COPD.

Gene Product	***Putative Function(s)***
GILZ	*GILZ* is a glucocorticoid-inducible gene that inhibits the transcriptional activity of both NF-κB and AP-1. Glucocorticoid-induced *GILZ* expression may also be modestly enhanced by LABAs. It is reported that induction of this gene suppresses various indices of inflammation (Mittelstadt and Ashwell, 2001).
RGS2	RGS2 is a GTPase-activating protein that switches off signalling from Gq-linked GPCRs. In airway smooth muscle, *RGS2* is up-regulated in a positive cooperative manner by glucocorticoid and LABA and exerts a bronchoprotective effect; in epithelial and other cells, RGS2 expression may attenuate pro-inflammatory mediator release (Holden et al., 2013).
CD200	Pulmonary alveolar macrophages have high constitutive expression of CD200R. Signaling through this receptor involves the interaction of CD200R-bearing cells with other cells (e.g. airway epithelia) that express the glucocorticoid-inducible, cognate agonist, CD200, which can be enhanced by formoterol and roflumilast alone and in combination. Studies in mice have shown that the CD200/CD200R interaction blunts macrophage activation measured as pro-inflammatory cytokine generation. Acute exacerbations of COPD are triggered, primarily, by prolonged bouts of excessive inflammation in response to bacterial and viral infections. Pharmacological up-regulation of *CD200* on epithelial and other airway cells in COPD could attenuate inflammation and reduce exacerbation frequency (Snelgrove et al., 2008).
CRISPLD2	Previously known to mediate several other functions, *CRISPLD2* was recently found to encode as a novel, secreted, mammalian LPS-binding protein in both humans and mice. Enhancement of FP-induced *CRISPLD2* expression could contribute to the reduction in exacerbations in COPD produced by infections with gram negative bacteria by down-regulating TLR4-mediated pro-inflammatory responses (Wang et al., 2009).
p57*kip2*	*p57*^{kip2} encodes a cell cycle kinase inhibitor that is induced by glucocorticoids in airway epithelial and other structural cells by a mechanism that is enhanced by formoterol and roflumilast, alone and in combination. Expression of this gene in relevant cells could arrest mitogenesis and suppress airway remodeling, which is a characteristic feature of COPD. *p57*^{kip2} may also block pro-inflammatory responses through its ability to inhibit one of the core mitogen-activated protein kinases, *c-jun*-N-terminal kinase (Chang et al., 2003, Samuelsson et al., 1999).

Figure 1

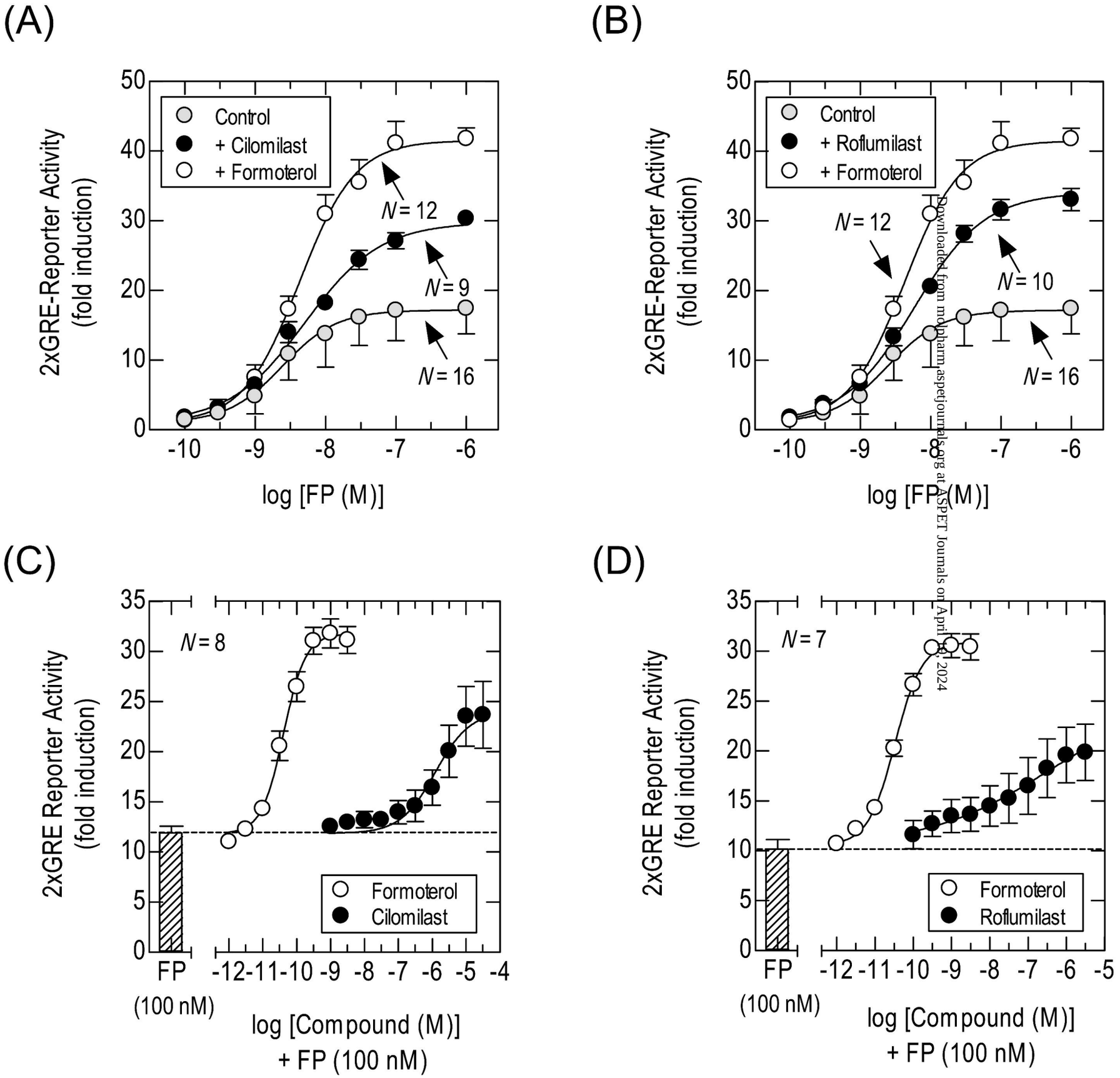

Figure 2

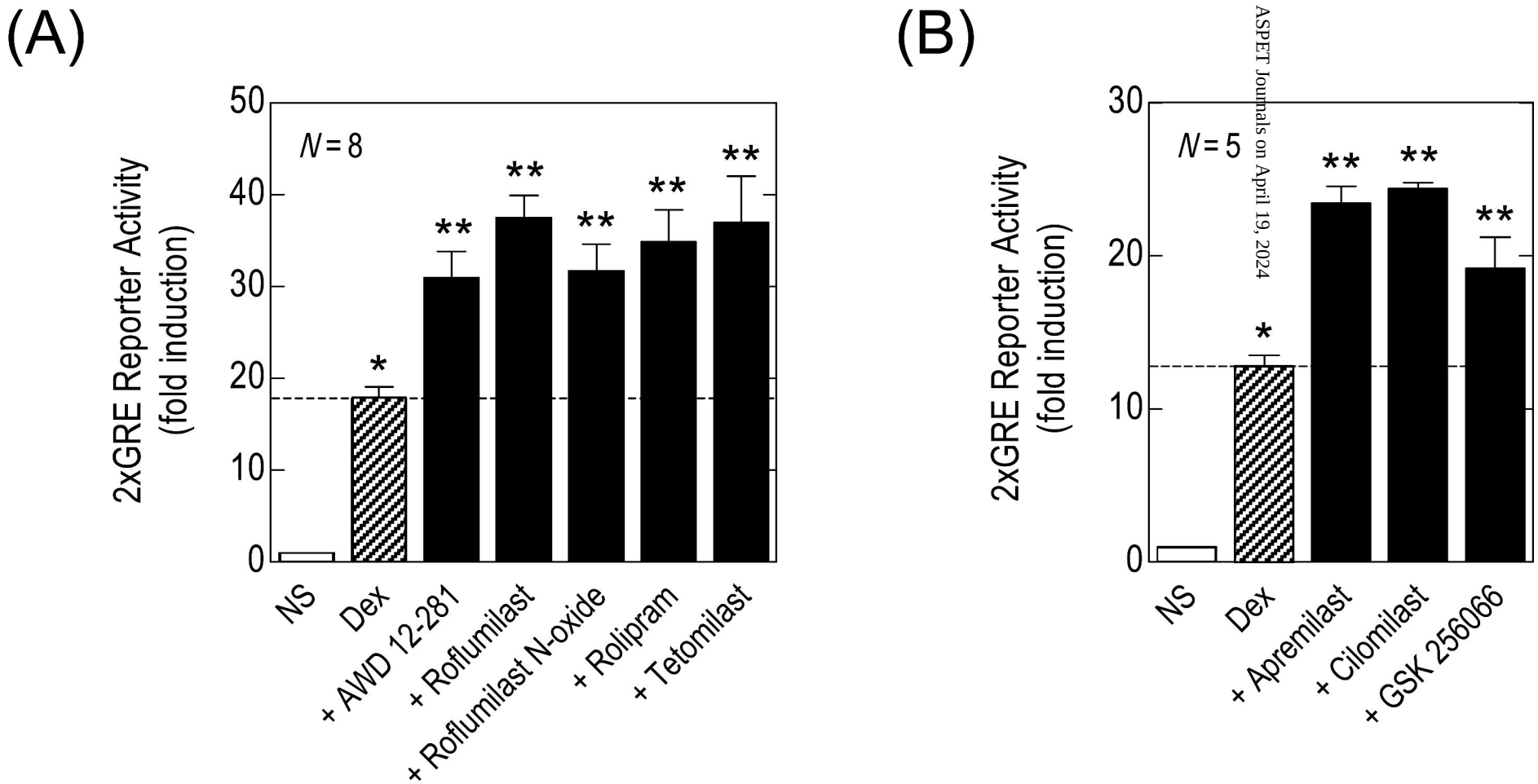

Figure 3

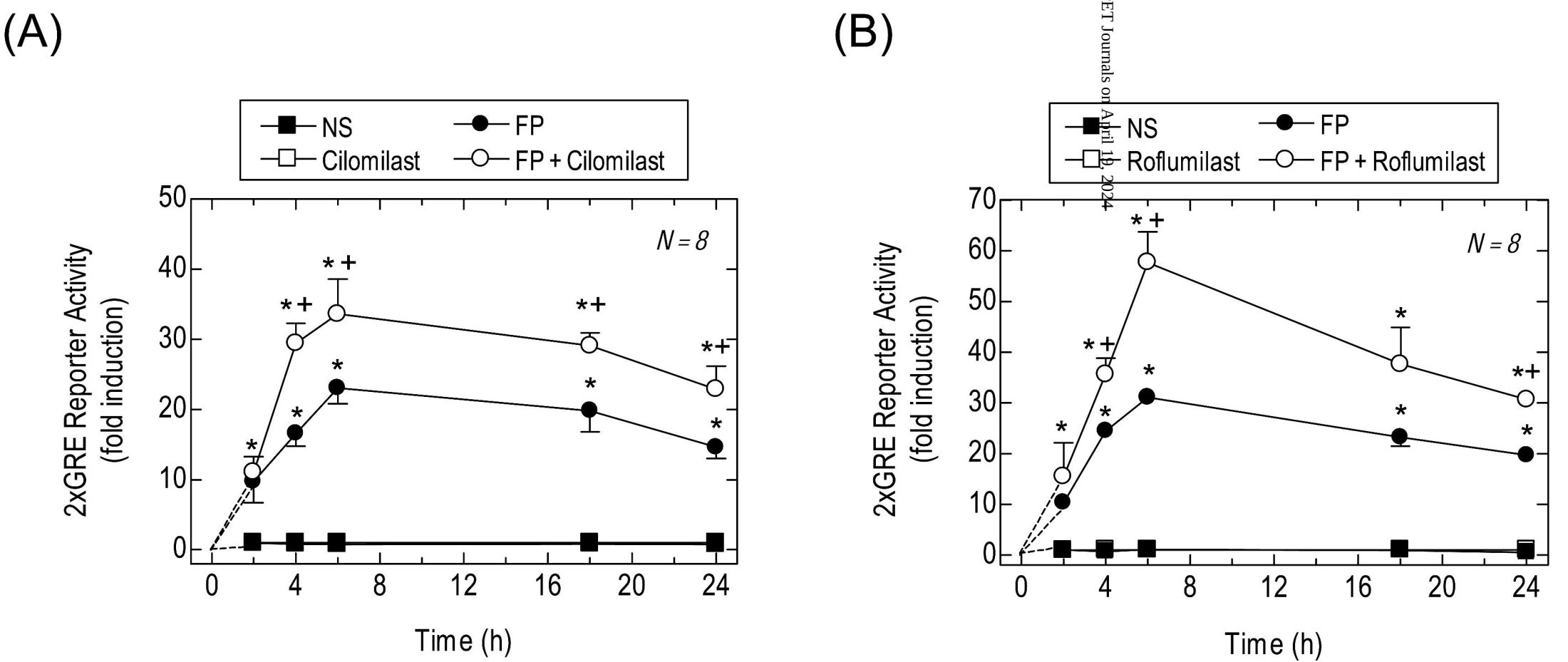

Figure 4

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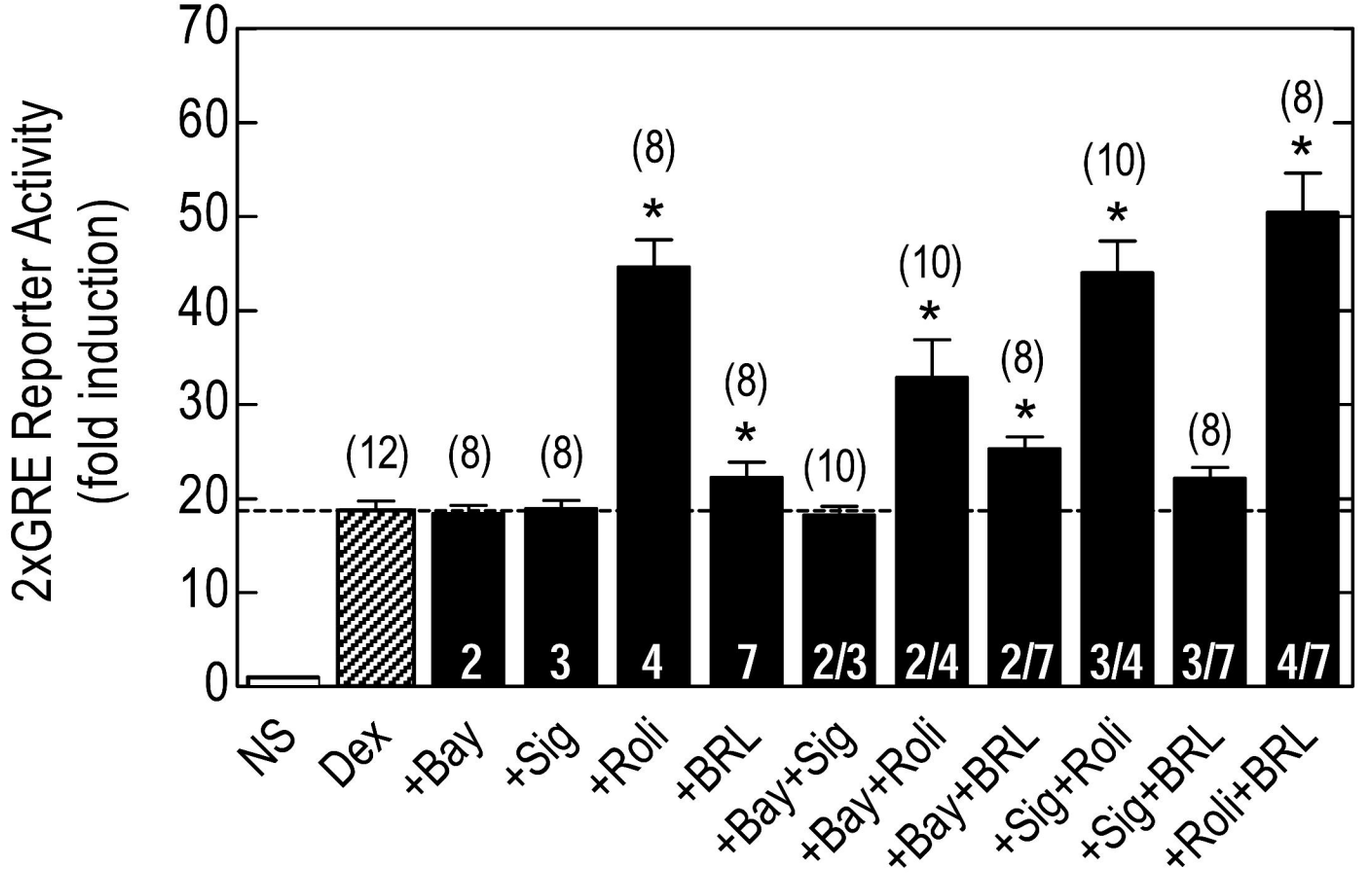

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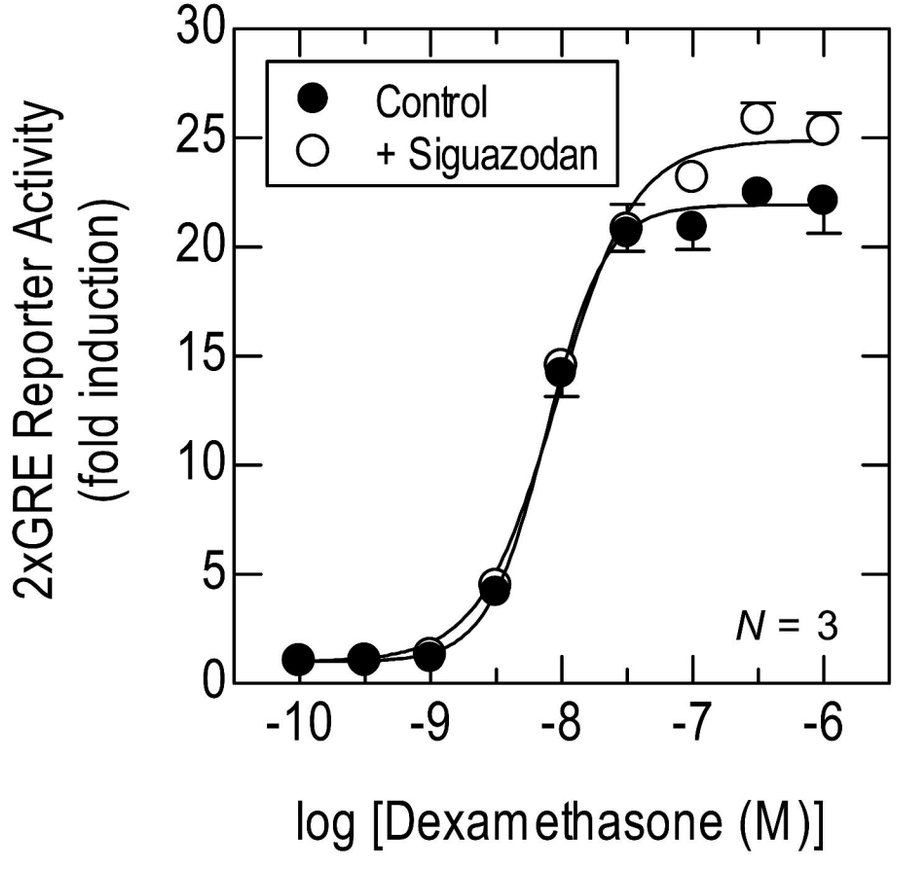


Figure 5

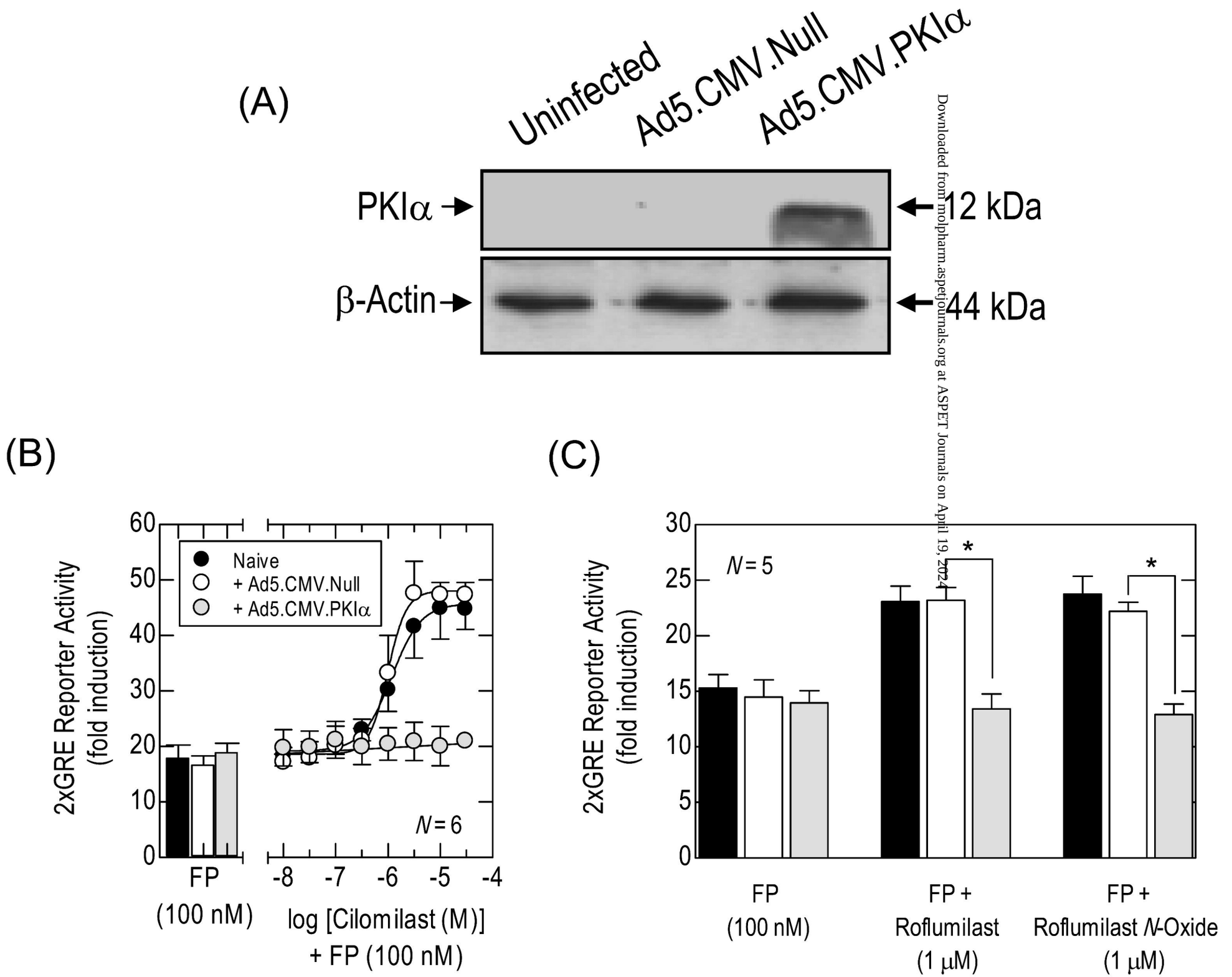


Figure 6

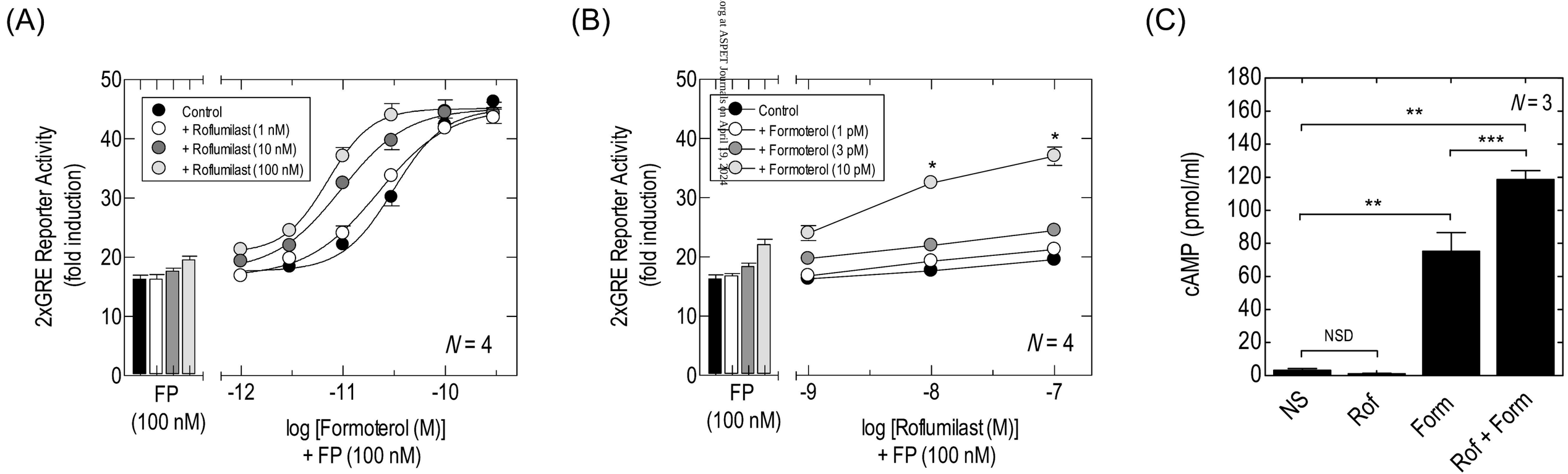


Figure 7

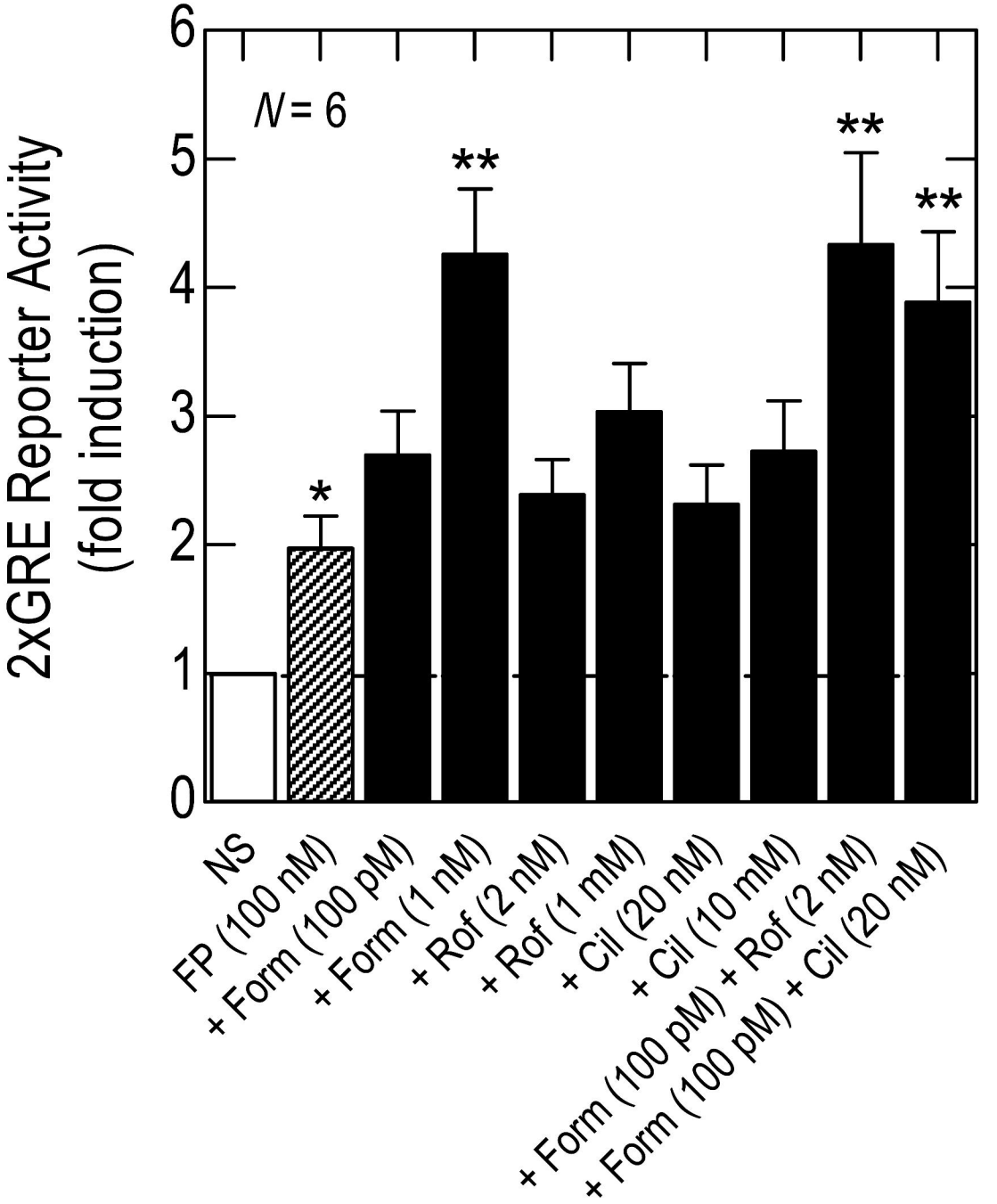

2xGRE Reporter Activity
(fold induction)
N = 6
0
1
2
3
4
5
6
*
**
**
**
NS
FP (100 nM)
+ Form (100 pM)
+ Form (1 nM)
+ Rof (2 nM)
+ Rof (1 mM)
+ Cil (20 nM)
+ Cil (10 mM)
+ Form (100 pM) + Rof (2 nM)
+ Form (100 pM) + Cil (20 nM)

Figure 8

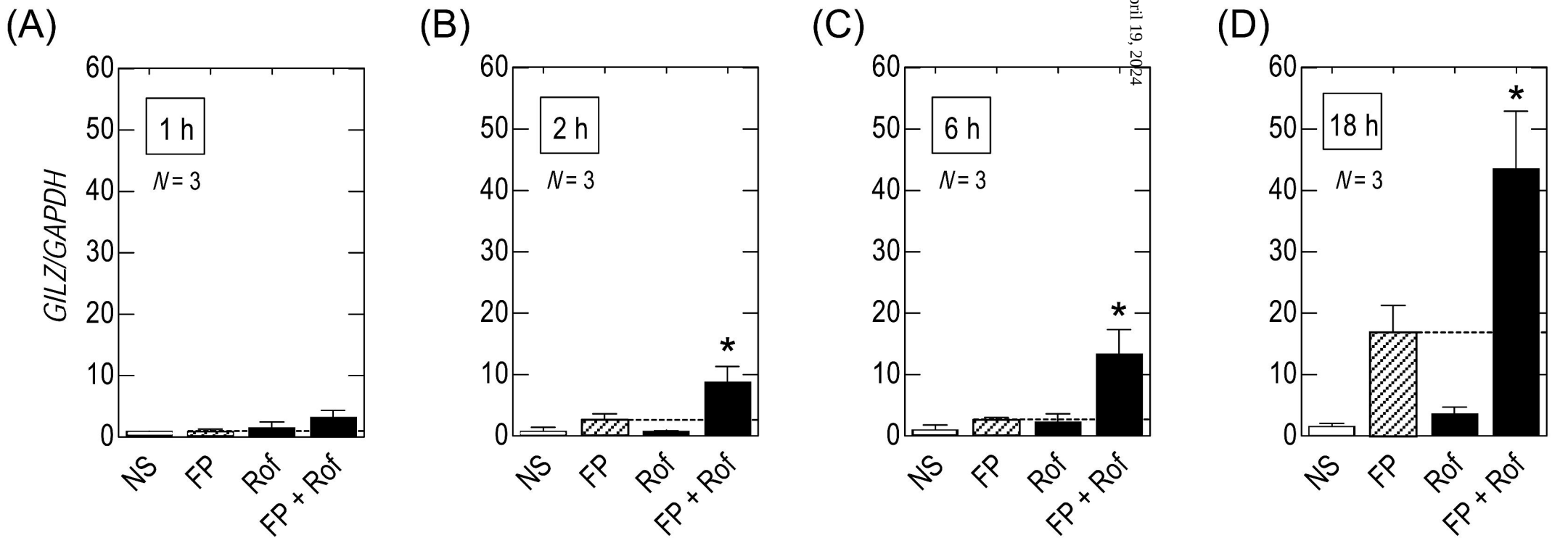


Figure 9

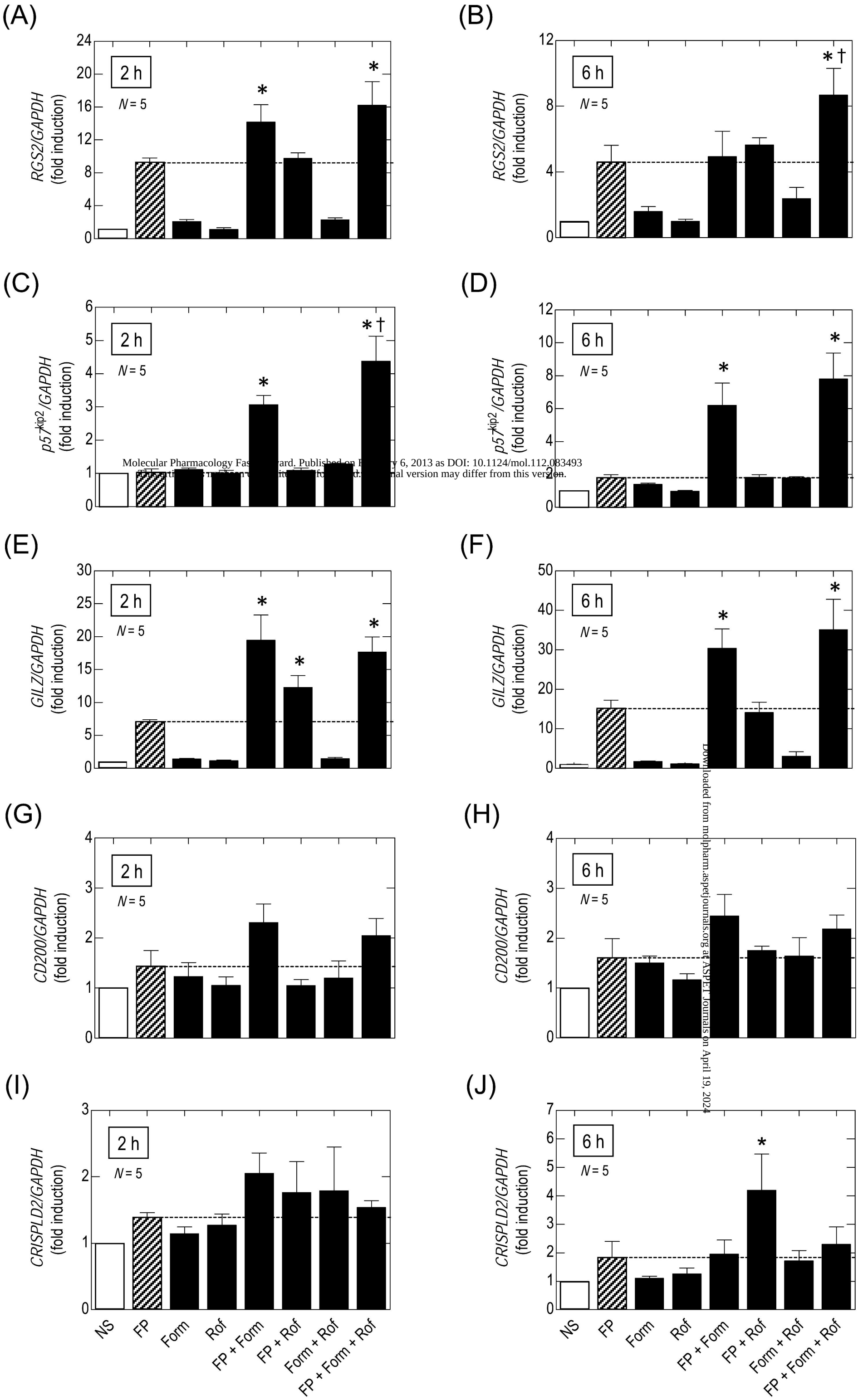
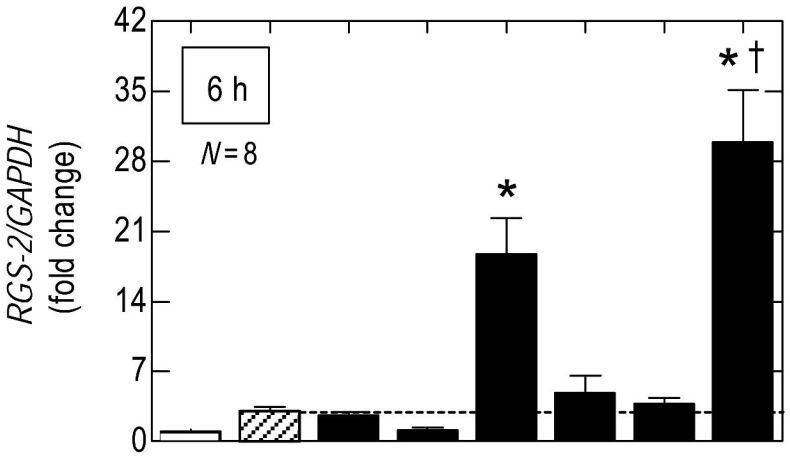


Figure 10

(A)



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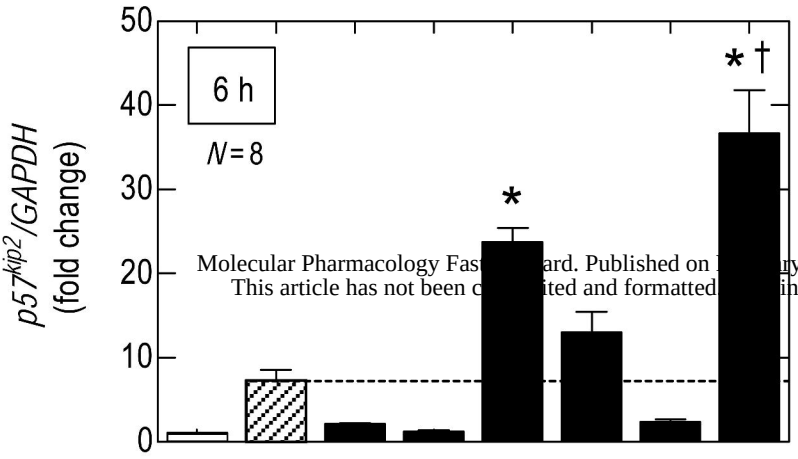

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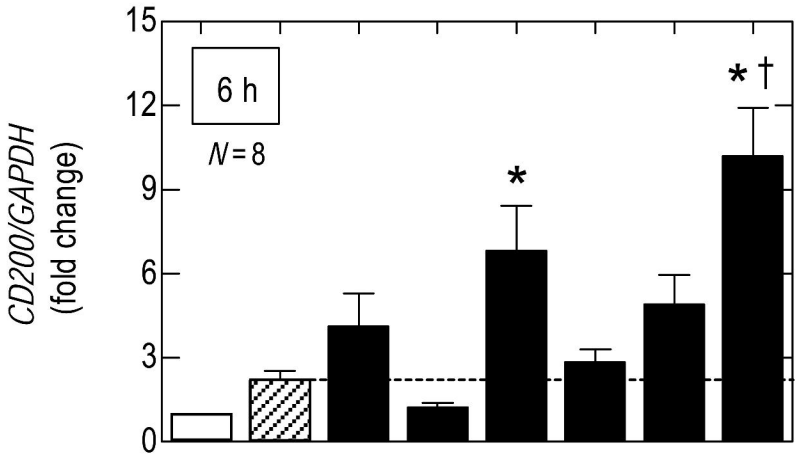

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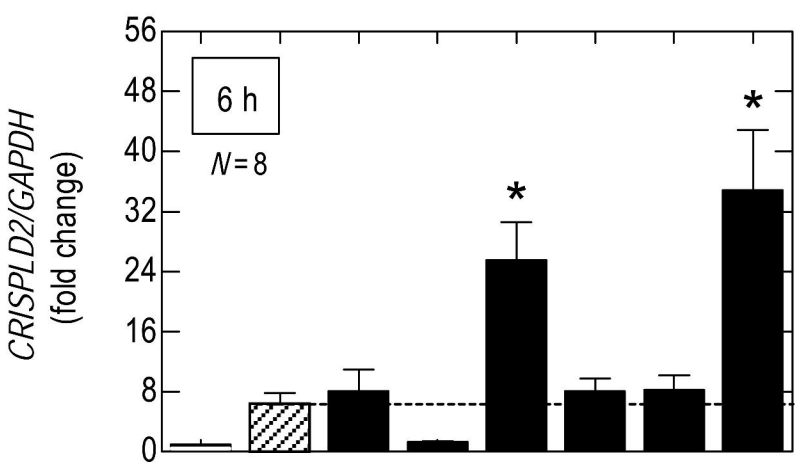

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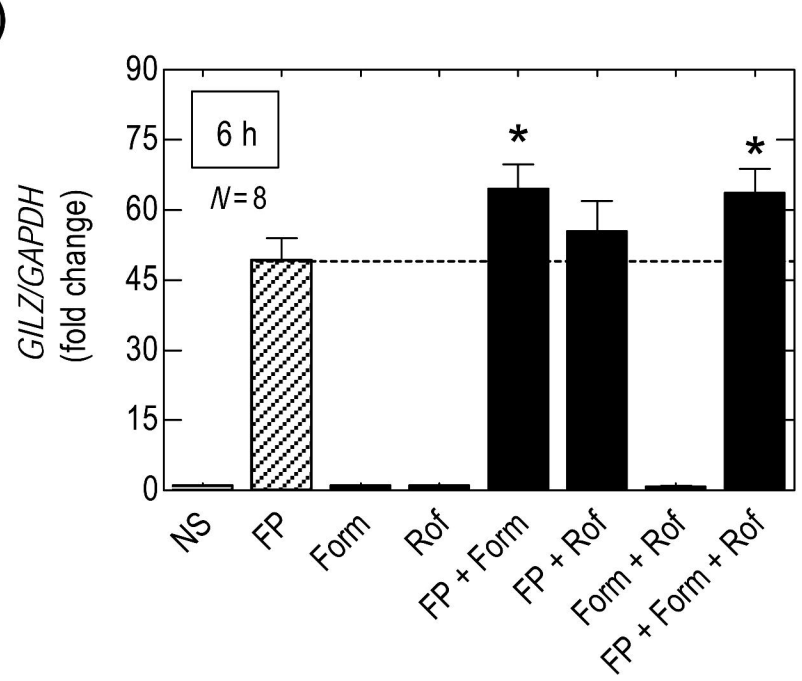

Figure 11