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Cyclic AMP-independent activation of *CYP3A4* gene expression by forskolin

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Abstract

Forskolin and cAMP have been shown to have paradoxical effects in the regulation of expression levels of mRNA of cytochrome *P*450 3A (CYP3A) family members. We demonstrate in this study that forskolin upregulated the promoter for CYP3A4 independent of its ability to increase cAMP levels. This activity was explained showing forskolin directly activated the pregnane-X-receptor, a known regulator of *CYP3A* genes.

Keywords: Pregnane-X-receptor; Forskolin; CYP3A

1. Introduction

Forskolin is a diterpene natural product widely used to help dissect cellular signaling pathways both in vitro and in vivo due to its potent activity as a stimulator of adenylate cyclase and subsequent elevation of intracellular cAMP (Seamon and Daly, 1986). Although most biological activities of forskolin can be explained through phosphorylation of cAMP-response element binding (CREB) protein, a transcription factor that modulates genes containing cAMP-response elements (CRE), several responses cannot be reproduced with cAMP analogs suggesting cAMP-independent mechanisms of action. Examples include antagonism of the effects of brefeldin A on the Golgi apparatus (Lippincott-Schwartz et al., 1991), inhibition of voltage-sensitive channel proteins (Hoshi et al., 1988), and inhibition of glucose transport through binding to the glucose transporter (Sergeant and Kim, 1985).

Several studies have examined the modulation of cytochrome *P*450 gene (*CYP*) expression by cAMP levels in hepatocytes (Sidhu and Omiecinski, 1995; Bani et al., 1998; Joannard et al., 2000; Marc et al., 2000). Understanding this regulation is important as family members are responsible for oxidative metabolism of a wide variety of structurally diverse compounds including approximately 60% of clinically marketed drugs (Maurel, 1996) as well as endogenous steroids and bile acids (Kliewer et al., 1998; Staudinger et al., 2001; Xie et al., 2001). Forskolin has been demonstrated to reduce expression of some *CYP* family members but increased *CYP3A1* mRNA levels (Sidhu and Omiecinski, 1995). However, non-hydrolysable cAMP analogs resulted in a reduction of phenobarbital-stimulated *CYP3A1* mRNA expression in rat hepatocytes, indicating that the activity of forskolin in inducing p4503A is independent of adenylate cyclase.

The mRNA expression of the cytochrome *P*450 3A subfamily was recently shown to be regulated by the pregnane-X-receptor (PXR, SXR, and NR1I2) (Kliewer et al., 1998). This receptor is capable of binding a diverse array of natural products and xenobiotics including lipophilic environmental contaminants and drugs. The receptor binds as a heterodimer with 9-*cis* retinoic acid receptor to the xenobiotic response element in *CYP3A* and other promoters

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to activate gene transcription. In this study, we provide evidence that forskolin acts as a direct pregnane-X-receptor ligand to activate *CYP3A4* expression independent of effects on adenylate cyclase.

2. Materials and methods

2.1. Materials

Affinity-purified, 6-His-tagged, full-length pregnane-X-receptor was from PanVera; cell culture materials were obtained from Biowhittaker except for charcoal/dextran-treated fetal bovine serum (FBS; Hyclone); forskolin, 8-bromo-cAMP, BSA, and other chemicals (Sigma); pGL5*luc* and pGL3-Basic plasmids (Promega, Corp.). pBlueScript II was from Stratagene; pCMV6-XL4 was from OriGene; coenzyme A and Fugene transfection reagent were from Roche; luciferin was from Biosynth; the human hepatoma cell line, HuH7, and human embryonic kidney cell line, HEK293, were from ATCC; low-volume 384-well assay plates were from Greiner; AlphaScreen beads and AlphaQuest HTS instrument were from Perkin-Elmer Life Sciences; and the NorthStar Imager was from Applied Biosystems.

2.2. Transfection assays

HuH7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% charcoal/dextran-treated FBS, 2 mM L-glutamine, and 20 mM Hepes. For Gal4 transfection assays, cells were trypsinized, counted, and transfected with 70 ng of GAL4-pregnane-X-receptor, 2 μg of pGL5Luc, 8 μg of pBlueScript II, and 21 μl of Fugene per 10^6 cells according to the manufacturer's directions in a 225-cm^2 flask. After incubation for 24 h at 37 °C in a 5% CO_2, cells were trypsinized and plated in 96-well cell culture plates at 50,000 cells/well. Following a 2–3 h attachment period, cells were treated with test compound in dimethyl sulfoxide (DMSO) (final DMSO concentration was 0.25%) for 24 h, cells lysed with luciferase lysis buffer, and plates read immediately in a NorthStar Imager. For the *CYP3A4* promoter assay, the human promoter region from −1192 to −6 was amplified by polymerase chain reaction (PCR) from a human genomic library and cloned into the pGL3-Basic reporter plasmid creating pGL3-CYP3A4-Prom. HEK293 cell line was maintained in 3:1 DMEM:F12 with 10% charcoal/dextran-treated FBS, 2 mM L-glutamine, and 20 mM Hepes and transfected with 5 μg of pCMV6-XL4- pregnane-X-receptor, 5 μg of pGL3-CYP3A4-Prom, and 10 μl of Fugene per 10^6 cells according to the manufacturer's directions in a 225-cm^2 flask. The remainder of the assay was as described above for the GAL4-pregnane-X-receptor assay.

2.3. Steroid receptor coactivator-1 preparation

The region encoding the nuclear receptor interacting domain of human steroid receptor coactivator-1 (SRC-1) (amino acids 220–394) was amplified by PCR and inserted into an *Escherichia coli* expression vector resulting in an NH_2-terminal, glutathione *S*-transferase fusion protein. Recombinant glutathione *S*-transferase-SRC-1 was expressed in BL21 (DE3) and purified protein produced by cell lysis and affinity chromatography on a glutathione affinity column.

2.4. Coactivator recruitment assay

Pregnane-X-receptor (45 nM) and SRC-1 (7.5 nM) in assay buffer (50 mM Hepes, pH 7.0; 150 mM NaCl; 0.1% BSA; 2 mM dithiothreitol) containing 15 μg/ml anti-glutathione *S*-transferase AlphaScreen acceptor beads and 15 μg/ml nickel chelate AlphaScreen donor beads were pipetted at 6 μl/well in low-volume, 384-well assay plates. Three microliters of compound or control solvent diluted in assay buffer was added to each well, the plates incubated at RT for 3 h in the dark, and read on an AlphaQuest HTS reader.

3. Results

3.1. Forskolin but not 8-bromo cAMP activates CYP3A4 promoter activity

We evaluated the ability of forskolin to activate a luciferase reporter gene driven by the 1.1 kb upstream regulatory region of CYP3A4 that includes the ER6 motif that binds pregnane-X-receptor/9-*cis* retinoic acid receptor heterodimer (pGL3-CYP3A4Prom). HEK293 cells were transiently transfected with pGL3-CYP3A4Prom and a pregnane-X-receptor expression plasmid, pCMV6-XL4-pregnane-X-receptor. Transfection of reporter alone yielded no response to the known CYP3A4 agonist, rifampicin or forskolin (Fig. 1A). In the presence of pregnane-X-receptor, rifampicin showed a concentration-dependent increase in luciferase activity demonstrating that the activity is mediated by pregnane-X-receptor. The cAMP analog 8-bromo-cAMP had no activity under either condition, consistent with the lack of CRE in the *CYP3A4* promoter. Forskolin, however, showed a clear concentration-dependent response in the presence of pregnane-X-receptor with an EC_{50} of 10 μM, suggesting that forskolin may be a ligand for pregnane-X-receptor. Interestingly, forskolin showed twice the efficacy as rifampicin.

To rule out forskolin activation of pregnane-X-receptor through binding to heterodimerization partner 9-*cis* retinoic acid receptor, we determined its ability to activate a GAL4 two-hybrid reporter system not dependent on 9-*cis* retinoic acid receptor in HuH7 cells. The pregnane-X-receptor

ligand-binding domain was fused to yeast GAL4 DNA-binding domain, creating pGL5-GAL4-pregnane-X-receptor-ligand binding domain. Pregnane-X-receptor agonists are detected by activation of a luciferase reporter gene under the control of a promoter containing five GAL4 binding sites (pGL5*luc*). Fig. 1B demonstrates that forskolin and rifampicin, but not 8-bromo-cAMP or the 9-*cis* retinoic acid receptor ligand 9-*cis* retinoic acid, activated luciferase expression in this system.

3.2. Forskolin stimulates pregnane-X-receptor recruitment of SRC-1

To measure the binding of forskolin to pregnane-X-receptor, we used a functional biochemical coactivator recruitment assay. Binding of agonists to nuclear receptors results in conformational change, increasing binding affinity for coactivator proteins (Onate et al., 1995). Pregnane-X-receptor has been shown to recruit a number of different

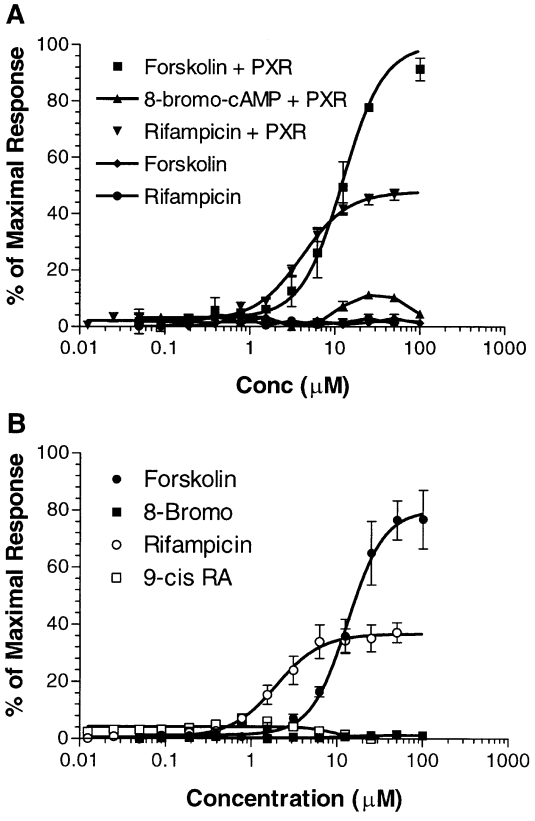

Fig. 1. Activity of compounds in pregnane-X-receptor reporter gene assays. (A) Concentration–response curves of forskolin (EC_{50}=12.4 μM, n=8), 8-bromo-cAMP, or the positive control rifampicin (EC_{50}=4.1 μM, n=8) in HEK293 cells transiently transfected with pGL3-CYP3A4-Prom with or without pCMV6-XL4-pregnane-X-receptor (PXR). Data were calculated relative to the largest response seen in the assay; no normalization to other genes was used. Symbols represent means and vertical lines represent the standard deviation of the mean. (B) Concentration–response curves of forskolin (EC_{50}=13.2 μM, n=8), 8-bromo-cAMP, 9-*cis*-retinoic acid, or the positive control rifampicin (EC_{50}=2.0 μM, n=8) in HuH7 cells transiently transfected with GAL4-pregnane-X-receptor and pGL5Luc.

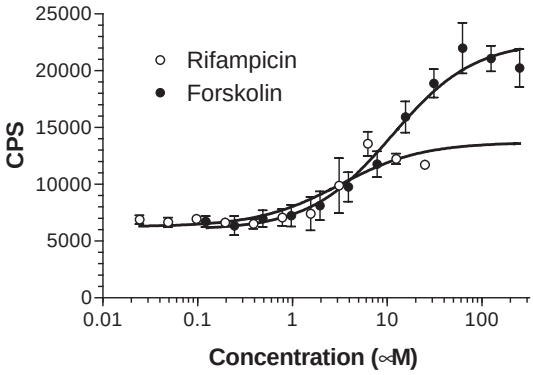

Fig. 2. Recruitment of coactivator SRC-1 by pregnane-X-receptor in response to agonists measured in an AlphaScreen™ format. Concentration–response curves to forskolin (EC_{50}=11.3 μM, n=4) and rifampicin (EC_{50}=3.3 μM, n=4); higher concentrations of rifampicin were removed from the calculation because of quenching of the AlphaScreen™ signal. Symbols represent means and vertical lines represent the standard deviation of the mean. *Y*-axis units are AlphaScreen™ counts per second (cps).

coactivators including the p160/SRC family in mammalian two-hybrid systems (Synold et al., 2001). Using a glutathione *S*-transferase fusion of a truncated construct of SRC-1 containing three nuclear receptor interacting domains, recruitment of glutathione *S*-transferase-SRC-1 to pregnane-X-receptor was measured in a homogeneous protein:protein interaction assay with AlphaScreen™ technology (Rouleau et al., 2003). Fig. 2 demonstrated that forskolin induced a concentration-dependent increase in SRC-1 recruitment. The EC_{50} obtained, 11 μM, was consistent with that seen in the cellular reporter assays, providing strong evidence that forskolin is a direct ligand for pregnane-X-receptor. Increased efficacy relative to rifampicin was noted, consistent with the cellular reporter data.

4. Discussion

Elevated levels of cAMP levels have been shown to negatively regulate levels of *CYP3A* and *CYP2B* gene expression (Sidhu and Omiecinski, 1995). Paradoxically, forskolin, whose classic pharmacological response is elevation of cAMP through activation of adenylate cyclase, has been shown to enhance *CYP3A1* but not *CYP2B* gene expression in primary rat hepatocytes (Sidhu and Omiecinski, 1996). The present study sought to resolve this paradox by determining the ability of forskolin to act as an agonist for the xenobiotic nuclear receptor pregnane-X-receptor, a known regulator of *CYP3A* expression. In both cellular reporter assays and an in vitro functional assay, forskolin behaved as a pregnane-X-receptor ligand with an EC_{50} of approximately 10 μM. Thus, despite well-characterized pharmacology associating forskolin actions with adenylate cyclase activation and elevation of cAMP, care must be take in interpreting results obtained with forskolin. Several unrelated protein targets of forskolin that are cAMP-independent have now been identified (Mandla and Tenen-

house, 1992). Indeed, the activity demonstrated in this study, as an agonist for the human xenobiotic nuclear pregnane-X-receptor, occurs in a system also modulated by cAMP levels, potentially confounding results. In our assay, using the transiently transfected CYP3A4 promoter controlling luciferase expression, forskolin has a dominant effect in activating the promoter through pregnane-X-receptor. However, under different cellular conditions (e.g., reduced concentrations of pregnane-X-receptor or coactivator proteins), it is possible that cAMP response would dominate.

The diterpene structure of forskolin fits the general model for ligand binding to pregnane-X-receptor that includes the common features of a largely hydrophobic core with a small number of polar groups capable of hydrogen bonding (Lehmann et al., 1998). The promiscuity of the ligand-binding pocket has been described both pharmacologically (Lehmann et al., 1998) and structurally (Watkins et al., 2001). The diterpene core is very analogous to the A, B, and C rings of steroids, known pregnane-X-receptor agonists (Kliewer et al., 1998). Spacing of hydrophobic and polar residues matches predictions from a pharmacophore model (Ekins et al., 2001) and a crystal structure model (Watkins et al., 2001). Forskolin joins a growing list of natural product substances that act as ligands for nuclear hormone receptors including guggul, an antagonist for farnesoid X-receptor extracted from the resin of the Guggul tree (Urizar et al., 2002); the alkaline fungal metabolite paxilline, from *Penicillium paxilli*, an agonist for the liver X-receptors (Bramlett et al., 2003); and genestein, found in soybeans, with agonist activity for estrogen receptors (Kuiper et al., 1998) and peroxisome proliferator receptor γ (Dang et al., 2003).

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