In vitro PKA phosphorylation-mediated human PDE4A4 activation

France Laliberté, Susana Liu, Elise Gorseth, Brian Bobechko, Adrienne Bartlett, Paula Lario, Michael J. Gresser, Zheng Huang*

Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutical Research, P.O. Box 1005, Pointe Claire, Dorval, QC, Canada H9R4P8

Received 30 November 2001; accepted 23 December 2001

First published online 16 January 2002

Edited by Judith Ovádi

Abstract The PDE4 catalytic machinery comprises, in part, two divalent cations in a binuclear motif. Here we report that PDE4A4 expressed in Sf9 cells exhibits a biphasic Mg^{2+} dose–response (EC_{50} of ~0.15 and >10 mM) in catalyzing cAMP hydrolysis. In vitro phosphorylation of PDE4A4 by the PKA-catalytic subunit increases the enzyme's sensitivity to Mg^{2+}, leading to 4-fold increased cAMP hydrolysis without affecting its K_m. The phosphorylation also increases the potencies of (*R*)- and (*S*)-rolipram without affecting CDP-840 and SB-207499. The results support that modulating the cofactor binding affinity of PDE4 represents a mechanism for regulating its activity. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: PDE4; PKA; Phosphorylation; Mg^{2+}; Rolipram

1. Introduction

3′,5′-Cyclic nucleotide phosphodiesterases (PDEs) are metallohydrolases that catalyze the hydrolysis of cAMP and cGMP. In concert with multiple adenylate cyclases, 11 families of PDEs regulate cAMP and cGMP-mediated signaling in specific tissues and subcellular locations [1]. The cAMP-specific PDE4s, encoded by four genes (A–D), are the major regulator of cAMP metabolism in most inflammatory and immune cells [2–4]. PDE4 inhibitors are efficacious in clinic trials for the treatment of asthma and chronic obstructive pulmonary disease (COPD) [4–6].

Each of the four PDE4 genes encodes multiple proteins that share identical catalytic and C-terminal domains but differ near their N-termini. Two upstream conserved regions (UCR1 and UCR2) are located between the divergent N-termini and the catalytic domains of the longer variants. Shorter variants lack the UCR1 domain [3]. The divergent N-termini contain signals for compartmentalization and protein/protein interactions, thereby allowing the fine-tuning of cAMP signaling to discrete subcellular locations and specific pathways [2,3]. This is exemplified by the identification of the PDE4/anchoring protein/PKA signaling complexes [7,8].

Intracellular cAMP concentrations fluctuate transiently within a narrow range upon receptor stimulation [9]. In addition to desensitization at the receptor and cyclase levels, phosphorylation-mediated PDE activation plays a pivotal role in regulating the transient changes of cAMP concentrations [10–15]. Elevated PDE3 and PDE4 activities, resulting from PKA activation, have been implicated in the short-term feedback regulation of cAMP concentrations in platelets, adipocytes, myoblasts, aortic smooth muscle cells, osteoclasts and U937 cells, indicating the ubiquitous nature of the mechanism [16–23]. At the molecular level, phosphorylation of the conserved RRES motif in UCR1 is responsible for the PKA-mediated activation of PDE4D3 by relieving an inhibitory constraint from the UCR1 and UCR2 domains on the catalytic machinery [18,24–27]. An Erk2-mediated phosphorylation of the conserved SPS motif, which lies at the C-terminal of the PDE4 catalytic domain, regulates the activities of PDE4B, 4C and 4D differentially [28–30]. Structurally, a tightly bound M_1 (likely Zn^{2+}) and a readily exchangeable M_2 ion (Mg^{2+} preferred) in a binuclear motif constitute the central feature of PDE4 catalytic machinery [31]. The metal ions are responsible for eliciting the high affinity cAMP binding, providing the hydroxyl nucleophile and facilitating AMP release from the active site [31–33]. An active site conformational difference in the presence and absence of the cofactor ions leads to the differential binding of inhibitors including the high and low affinity binding of 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrolidinone (rolipram). Both cofactors of the holoenzyme (M_1/M_2-bound PDE4) appear to be involved in mediating the high affinity rolipram/PDE4 interaction. In their absence, rolipram binds to the PDE4 apoenzyme with a K_d ~150 nM [33]. The involvement of the metal ions in PDE4 catalysis suggests that modulating the cofactor binding affinity represents a mechanism for its activity regulation [6,24,32,33]. This is supported by a biphasic Mg^{2+} response and an increased Mg^{2+} sensitivity upon the Ser^{54} phosphorylation of PDE4D3 [18,24,26].

Herein we report that human PDE4A4 expressed and partially purified from Sf9 cells also exhibits a biphasic response to Mg^{2+}. PKA-mediated further phosphorylation of PDE4A4 results in a 4-fold increased activity, resulting from an increased Mg^{2+} sensitivity. In addition, PDE4A4 phosphorylation selectively increases the sensitivity of the enzyme to both enantiomers of rolipram.

*Corresponding author. Fax: (1)-514-428-4900.
E-mail address: zheng_huang@merck.com (Z. Huang).

Abbreviations: BAEE, *N*-α-benzoyl-L-arginine ethyl ester; CDP-840, (*R*)-(+)-4-(2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl) pyridine; DEAE, diethylaminoethyl–Sepharose; GST, glutathione-*S*-transferase; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; PDE4, type 4 cyclic nucleotide phosphodiesterase; Rolipram, 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrolidinone; SB-207499, *cis*-4-[cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-(*R*)-1-cyclohexane carboxylic acid; UCR, upstream conserved region

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)02259-7

2. Materials and methods

2.1. Chemicals

[^{3}H]cAMP, PDE-SPA™ beads and diethylaminoethyl–Sepharose (DEAE–Sepharose) (Fast Flow) were purchased from Amersham Pharmacia Biotech. The catalytic subunit of PKA was purchased from Boehringer Mannheim. PDE4 inhibitors were synthesized at Merck Frosst. Other chemicals were purchased from Sigma/Aldrich.

2.2. Expression and purification of PDE4A4

Human PDE4A4 was expressed using the baculovirus/Sf9 expression system and was partially purified on a DEAE-cation exchange column using a linear KCl gradient following procedures similar to that described previously [34,35]. Protein concentration was estimated with BSA as standard using the Bio-Rad protein assay kit.

2.3. PKA phosphorylation of PDE4A4

The partially purified PDE4A4 (40 μg) was incubated with the catalytic subunit of PKA (300 units) for 2 h at room temperature in a buffer (300 μl) containing 50 mM Tris (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 200 mM NaCl, 2 mM *N*-α-benzoyl-L-arginine ethyl ester (BAEE), 2 mM benzamidine, 0.2 mM DTT and 300 μM ATP. Phosphorylation was stopped by the addition of excess EDTA (15 mM) and the sample was stored at −80°C.

2.4. PDE activity assay

PDE4 activity was monitored by the hydrolysis of [^{3}H]cAMP to [^{3}H]AMP using the PDE-SPA kit from Amersham Pharmacia Biotech as described previously [32]. The assay mixture contained 100 nM [^{3}H]cAMP (1 μCi/ml) in a buffer containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 100 mM KCl and 2 μl of test compound in DMSO at 30°C. The reaction was initiated by the addition of enzyme for 10 min. The potency of inhibitors (IC_{50} value) was determined from an 11-point dose–response curve performed in duplicate. The Mg^{2+} dose–response was determined at 0.1 μM [^{3}H]cAMP using buffer treated with the K^+-form of the Chelex beads (Bio-Rad) to remove trace metal ions as previously described [34]. The ionic strength of the final assay solution was maintained at 150 mM using KCl with no specific ion effects observed for either K^+ or Cl^- ions.

2.5. Data analysis

Data were expressed as mean ± S.E.M. of $n \geq 3$ independent experiments unless otherwise specified. Dose–response curves and IC_{50} values were analyzed by a non-linear iterative regression routine using Grafit™ (Erithacus Software).

3. Results

3.1. PKA phosphorylation-mediated PDE4A4 activation

Human PDE4A4 was expressed using a baculovirus/Sf9 expression system and partially purified on a DEAE column. Upon elution with a linear KCl gradient, a single rolipram-sensitive PDE activity peak, paralleled by a single immunoreactive PDE4 band, was obtained with PDE4A4 accounting for ∼10% of the total proteins by SDS–PAGE analysis under denaturing condition (Coomassie stain). In vitro phosphorylation of the partially purified PDE4A4 was conducted using the PKA-catalytic subunit in the presence of 0.3 mM ATP and 10 mM $MgCl_2$. Fig. 1 shows its PDE activity–cAMP concentration response before (△) and after (○) PKA/Mg-ATP treatment at 0.5 mM Mg^{2+}. The apparent V_{max} value increased by 4-fold (from 0.2 to 0.8 μmol/mg/min) after the PKA/Mg-ATP treatment with a slightly reduced apparent K_m (from 3.2 to 2.2 μM). Incubation with either the catalytic subunit or ATP alone exerted negligible effect on the PDE activity. No PDE4A4 degradation was detected during the phosphorylation procedure by Western blot analysis. Phosphorylation of PDE4A4 was confirmed by the detection of a radiolabeled PDE4A4 using [^{32}P]ATP. In contrast to the reduced mobility observed with Ser^{54}-phosphorylated PDE4D3 on SDS–PAGE [26], PDE4A4 phosphorylated from the PKA/Mg-ATP treatment comigrated with PDE4A4 under a variety of conditions tested.

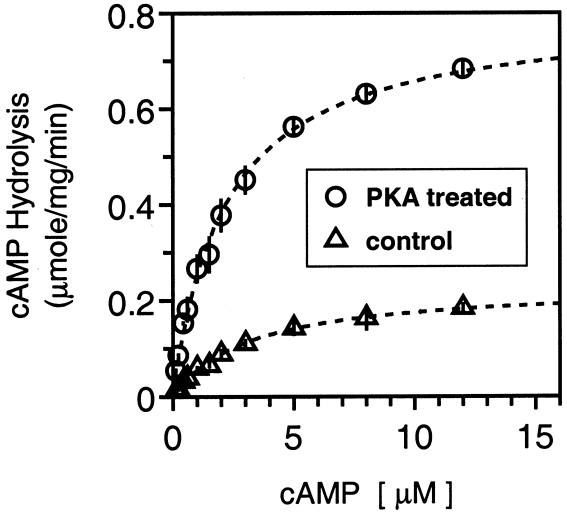

Fig. 1. PDE4A4-catalyzed cAMP hydrolysis at increasing cAMP concentration before and after PKA/Mg-ATP treatment. Hydrolysis of [^{3}H]cAMP at increasing cAMP concentrations by PDE4A4 before (△) and after (○) PKA/Mg-ATP treatment was determined in a buffer containing 20 mM HEPES (pH 7.5), 0.5 mM $MgCl_2$ and 100 mM KCl at room temperature. The values represent the mean (± S.E.M.) of three measurements. Saturation analysis yielded the K_m values of 3.2 (± 0.2) and 2.2 (± 0.2) μM before and after the PKA-mediated activation. The apparent V_{max} values were at 0.2 (± 0.03) and 0.8 (± 0.1) μmol/mg/min respectively for the partially purified PDE4A4 (∼10% pure from Coomassie stain on SDS–PAGE).

Fig. 2 shows the PDE activity–Mg^{2+} dose–response of

Table 1
Potency of inhibitors against PDE4A4 before and after PKA activation compared with their Mg^{2+}-dependent and -independent binding affinities against GST–PDE4A^{248}

Compound	Inhibition of catalysis (IC_{50}, nM)[a]		Binding affinity (IC_{50}, nM)[b]	
	PDE4A4	PDE4A4^{PKA}	(10 mM EDTA)	(5 mM Mg^{2+})
(*R*)-Rolipram	123 (± 30)	8 (± 3)	150 (± 37)	5 (± 1)
(*S*)-Rolipram	580 (± 50)	120 (± 15)	290 (± 68)	64 (± 4)
CDP-840	7 (± 2)	4 (± 2)	> 4000	6 (± 2)
SB-207499	39 (± 5)	45 (± 7)	> 4000	42 (± 7)

[a]The inhibitory potencies (mean ± S.E.M., $n \geq 3$) against the partially purified PDE4A4 before (PDE4A4) and after the PKA/Mg-ATP treatment (PDE4A4^{PKA}) were determined in a buffer containing 20 mM HEPES (pH 7.5), 1 mM EDTA, 10 mM Mg^{2+}, 100 mM KCl and 0.1 μM [^{3}H]cAMP.
[b]The binding affinities (mean ± S.E.M., $n \geq 3$) of inhibitors against GST–PDE4A^{248} in 5 mM free Mg^{2+} and in 10 mM EDTA were adapted from [33].

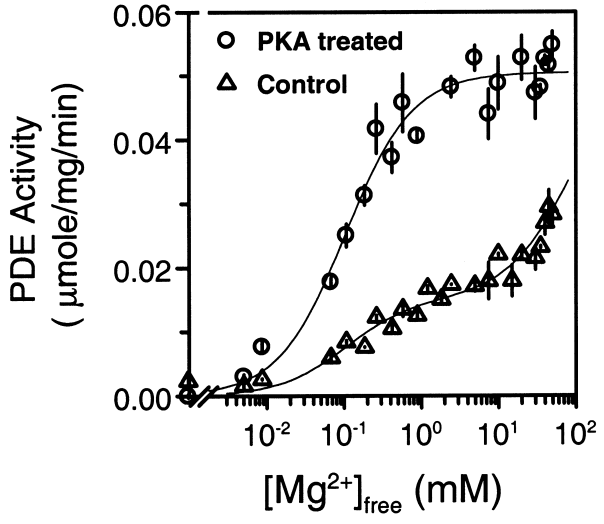

Fig. 2. PDE4A4-catalyzed cAMP hydrolysis at increasing Mg^{2+} concentrations before and after PKA/Mg^{2+}-ATP treatment. The rate of cAMP hydrolysis at increasing Mg^{2+} concentrations before (△) and after (○) PKA/Mg-ATP treatment of PDE4A4 was determined at 150 mM constant ionic strength in a buffer containing 0.1 μM [^{3}H]cAMP and 20 mM HEPES (pH 7.5) at room temperature. The values represent the mean (±S.E.M.) of two measurements. The monophasic Mg^{2+} response (○) has an EC_{50} value at 0.11 (±0.04) mM for Mg^{2+}. The biphasic Mg^{2+} response (△) of the control were simulated using a two binding site model with EC_{50} values at 0.15 (±0.05) and 100 (±20) mM respectively.

PDE4A4 before (△) and after (○) the PKA-mediated phosphorylation. The activity was detected using 0.1 μM cAMP under a constant ionic strength (150 mM) which minimizes the effect of ionic strength-induced activity changes [27]. Before the PKA/Mg-ATP treatment, the Mg^{2+} dose–response was biphasic with high and low affinity responses (EC_{50}) of ~0.15 mM and >10 mM respectively. Following phosphorylation, the Mg^{2+} dose–response became monophasic with an EC_{50} value of 0.11 (±0.04) mM, which resulted in ~4-fold increased PDE activity at Mg^{2+} concentrations below 10 mM. Thus, the PKA-mediated PDE4A4 phosphorylation increases the enzyme's sensitivity to Mg^{2+}-mediated activation, analogous to that of PDE4D3 [18,23,24].

3.2. Selective perturbation of inhibitor potency

Besides the increased rate of cAMP hydrolysis, in vitro phosphorylation of the PDE4A4 selectively increased the potency (IC_{50} value, inhibition of catalysis at 0.1 μM cAMP) of (*R*)-rolipram from 123 to 8 nM and of (*S*)-rolipram from 580 to 120 nM. In contrast, the potency of (*R*)-(+)-4-(2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl) pyridine (CDP-840) and *cis*-4-[cyano-4-(3-cyclopentyloxy-4-methoxyphenyl-(*R*)-1-cyclohexane carboxylic acid (SB-207499) were minimally perturbed by the enzyme phosphorylation (Table 1). The Mg^{2+}-dependent (5 mM Mg^{2+}) and independent (10 mM EDTA) components of PDE4 bindings of these inhibitors were recently delineated by the displacement of glutathione-*S*-transferase (GST)–$PDE4A^{248}$ ($HSPDE4A4B^{248-886}$) bound [^{3}H]rolipram in a SPA binding assay and they are listed in Table 1 for comparison [33]. After phosphorylation, the potencies of the dual binding rolipram enantiomers against PDE4A4 approached their holoenzyme binding affinities with GST–$PDE4A^{248}$. The potencies of the Mg^{2+}-dependent CDP-840 and SB-207499 were insensitive to the PKA-mediated PDE4A4 phosphorylation.

4. Discussion

The present study demonstrates that in vitro PKA-mediated phosphorylation of PDE4A4 activates the enzyme by increasing its sensitivity to the Mg^{2+} cofactor. In addition, the PDE4A4 phosphorylation selectively shifted the potencies of (*R*)- and (*S*)-rolipram, which bind to both apo- and holoenzyme, toward their holoenzyme binding affinities. Thus, the potency of the dual binding inhibitor rolipram is coupled to the enzyme activation state [32,33]. Although the PDE4 apoenzyme was detected under a stringent metal-free environment [35], M_1-bound PDE4, holoenzyme and their cAMP complexes likely dominate the distribution of enzyme species during catalysis. Rolipram binding to the M_1-bound PDE4 remains to be determined, which could further the understanding of its inhibition kinetics and phosphorylation sensitivity [33].

Several serines were phosphorylated in PDE4D3 upon PKA treatment, with the Ser^{54} phosphorylation of the RRES motif being responsible for its PKA-mediated cellular activation [18,24]. Phospho-PDE4A4 was detected from a ^{32}P-phosphate-supplemented medium, indicating that PDE4A4 expressed in Sf9 cells is at least partially phosphorylated (unpublished data). $PDE4A^{330-723}$ and GST–$PDE4A^{248}$ expressed in Sf9 cells under similar conditions are extensively phosphorylated at both serines of the SPS motif by mass spectral analysis [36]. Phospho-peptides that correspond to the phosphorylated RRES and SPS motifs were detected in a tryptic digestion mixture of the PDE4A4 by mass spectral analysis. However, the low purity of the enzyme preparation, coupled with the poor recovery of the phospho-peptides on mass spectrometer, has hampered the quantification of their phosphorylation level. A partially phosphorylated PDE4A4 provides the simplest explanation for the biphasic Mg^{2+} response in Fig. 2 and its sensitivity toward PKA-mediated further phosphorylation. Prolonged alkaline or acid phosphatase treatment of the partially phosphorylated PDE4A4 from Sf9 cells both shifted the (*R*)-rolipram potency to 200–500 nM. The resulting material after removing the phosphatases on an ion-exchange column (phosphatases interfere the PDE assay by hydrolyzing AMP) was unstable and exhibited a similar biphasic Mg^{2+} response with a much reduced and variable V_{max} value (up to 10-fold). The protein appears to be quite sticky. Under the same conditions, extensive dephosphorylations of the SPS motif were detected in GST–$PDE4A^{248}$ (unpublished data). Therefore, further studies are needed to dissect the effects of the multiple serine phosphorylation.

Previous studies indicate that the PKA-mediated Ser^{54} phosphorylation in PDE4D3 alleviates an electrostatically mediated inhibitory constraint on catalysis [25–27]. In addition to the activation by PKA, high ionic strength (>300 mM KCl) activates the partially purified PDE4A4 by 2–3-fold and increases the enzyme's sensitivity to (*R*)-rolipram, with its potency approaching the holoenzyme binding affinity in 800 mM KCl and 5 mM $MgCl_2$. Under the high ionic strength conditions, the PDE activity–Mg^{2+} response approaches monophasic (unpublished data). These data support the presence of a similar inhibitory constraint in PDE4A4. The binuclear ion center of PDE4 is located at the interface of three subdomains

[31]. It is tempting to speculate that the enzyme's cofactor binding affinity may be sensitive to perturbations within these domains. Modulating the cofactor binding affinity, through phosphorylations and protein/protein interactions, may represent a mechanism of PDE4 regulation.

Acknowledgements: We would like to thank Drs. Dave Percival and Brian Kennedy for many helpful discussions.

References

[1] Soderling, S.H. and Beavo, J.A. (2000) Curr. Opin. Cell Biol. 12, 174–179.
[2] Conti, M. and Jin, S.L. (1999) Prog. Nucl. Acid Res. Mol. Biol. 63, 1–38.
[3] Houslay, M.D. (2001) Prog. Nucl. Acid Res. Mol. Biol. 69, 249–315.
[4] Torphy, T.J. (1998) Am. J. Respir. Crit. Care Med. 157, 351–370.
[5] Bundschuh, D.S., Eltze, M., Barsig, J., Wollin, L., Hatzelmann, A. and Beume, R. (2001) J. Pharmacol. Exp. Ther. 297, 280–290.
[6] Huang, Z., Ducharme, Y., Macdonald, D. and Robichaud, A. (2001) Curr. Opin. Chem. Biol 5, 432–438.
[7] Tasken, K.A., Collas, P., Kemmner, W.A., Witczak, O., Conti, M. and Tasken, K. (2001) J. Biol. Chem. 276, 21999–22002.
[8] Dodge, K.L., Khouangsathiene, S., Kapiloff, M.S., Mouton, R., Hill, E.V., Houslay, M.D., Langeberg, L.K. and Scott, J.D. (2001) EMBO J. 20, 1921–1930.
[9] Brooker, G. (1973) Science 182, 933–934.
[10] Gettys, T.W., Blackmore, P.F., Redmon, J.B., Beebe, S.J. and Corbin, J.D. (1987) J. Biol. Chem. 262, 333–339.
[11] Corbin, J.D., Gettys, T.W., Blackmore, P.F., Beebe, S.J., Francis, S.H., Glass, D.B., Redmon, J.B., Sheorain, V.S. and Landiss, L.R. (1988) Methods Enzymol. 159, 74–82.
[12] Barber, R., Clark, R.B., Kelly, L.A. and Butcher, R.W. (1978) Adv. Cyclic. Nucleotide. Res. 9, 507–516.
[13] Cooper, D.M., Mons, N. and Karpen, J.W. (1995) Nature 374, 421–424.
[14] Houslay, M.D. and Milligan, G. (1997) Trends Biochem. Sci. 22, 217–224.
[15] Conti, M., Nemoz, G., Sette, C. and Vicini, E. (1995) Endocr. Rev. 16, 370–389.
[16] Corbin, J.D., Beebe, S.J. and Blackmore, P.F. (1985) J. Biol. Chem. 260, 8731–8735.
[17] Macphee, C.H., Reifsnyder, D.H., Moore, T.A. and Beavo, J.A. (1986) J. Cycl. Nucl. Protein Phosphor. Res. 11, 487–496.
[18] Oki, N., Takahashi, S.I., Hidaka, H. and Conti, M. (2000) J. Biol. Chem. 275, 10831–10837.
[19] Gettys, T.W., Vine, A.J., Simonds, M.F. and Corbin, J.D. (1988) J. Biol. Chem. 263, 10359–10363.
[20] Ekholm, D., Belfrage, P., Manganiello, V. and Degerman, E. (1997) Biochim. Biophys. Acta 1356, 64–70.
[21] Liu, H. and Maurice, D.H. (1999) J. Biol. Chem. 274, 10557–10565.
[22] Ball, E.H., Seth, P.K. and Sanwal, B.D. (1980) J. Biol. Chem. 255, 2962–2968.
[23] Alvarez, R., Sette, C., Yang, D., Eglen, R.M., Wilhelm, R., Shelton, E.R. and Conti, M. (1995) Mol. Pharmacol. 48, 616–622.
[24] Sette, C. and Conti, M. (1996) J. Biol. Chem. 271, 16526–16534.
[25] Hoffmann, R., Wilkinson, I.R., McCallum, J.F., Engels, P. and Houslay, M.D. (1998) Biochem. J. 333, 139–149.
[26] Lim, J., Pahlke, G. and Conti, M. (1999) J. Biol. Chem. 274, 19677–19685.
[27] Beard, M.B., Olsen, A.E., Jones, R.E., Erdogan, S., Houslay, M.D. and Bolger, G.B. (2000) J. Biol. Chem. 275, 10349–10358.
[28] Baillie, G.S., MacKenzie, S.J., McPhee, I. and Houslay, M.D. (2000) Br. J. Pharmacol. 131, 811–819.
[29] MacKenzie, S.J., Baillie, G.S., McPhee, I., Bolger, G.B. and Houslay, M.D. (2000) J. Biol. Chem. 275, 16609–16617.
[30] Hoffmann, R., Baillie, G.S., MacKenzie, S.J., Yarwood, S.J. and Houslay, M.D. (1999) EMBO J. 18, 893–903.
[31] Xu, R.X., Hassell, A.M., Vanderwall, D., Lambert, M.H., Holmes, W.D., Luther, M.A., Rocque, W.J., Milburn, M.V., Zhao, Y., Ke, H. and Nolte, R.T. (2000) Science 288, 1822–1825.
[32] Laliberte, F., Han, Y., Govindarajan, A., Giroux, A., Liu, S., Bobechko, B., Lario, P., Bartlett, A., Gorseth, E., Gresser, M. and Huang, Z. (2000) Biochemistry 39, 6449–6458.
[33] Liu, S., Laliberte, F., Bobechko, B., Bartlett, A., Lario, P., Gorseth, E., Hamme, J.V., Gresser, M. and Huang, Z. (2001) Biochemistry 40, 10179–10186.
[34] Perry, M.J., O'Connell, J., Walker, C., Crabbe, T., Baldock, D., Russell, A., Lumb, S., Huang, Z., Howat, D., Allen, R., Merriman, M., Walls, J., Daniel, T., Hughes, B., Laliberte, F., Higgs, G.A. and Owens, R.J. (1998) Cell Biochem. Biophys. 29, 113–132.
[35] Percival, M.D., Yeh, B. and Falgueyret, J.P. (1997) Biochem. Biophys. Res. Commun. 241, 175–180.
[36] Lario, P.I., Bobechko, B., Bateman, K., Kelly, J., Vrielink, A. and Huang, Z. (2001) Arch. Biochem. Biophys. 394, 54–60.