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Development of a Recombinant Cell-Based System for the Characterisation of Phosphodiesterase 4 Isoforms and Evaluation of Inhibitors

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ABSTRACT. We described the development of a recombinant cell-based system for the characterisation of phosphodiesterase (PDE) 4 isoforms and the evaluation of inhibitors. The Chinese hamster ovary (CHO) cell, which was found to have a low endogenous PDE4 background and no β-adrenergic receptors (β-AR), was transiently transfected with β-AR and various PDE4 isoforms which were expressed as functionally coupled molecules. From correlations of elevation of adenosine 3′,5′-cyclic monophosphate *in situ* and the inhibition of catalytic activity *in vitro* with the various PDE4 isoforms, it was apparent that PDE4A4, 4B2, 4C2, 4D2, and 4D3 all adopted a high-affinity binding conformation (i.e. expressed the high-affinity rolipram binding site) in the CHO cell, whereas PDE4A_{330} was expressed in a low-affinity conformation *in situ*. This gives the opportunity of using this system to screen and optimise inhibitors against a low-affinity conformation of PDE4 *in situ* and use a high-affinity conformation of PDE4 as a counterscreen, as inhibitor activity against this conformer has been linked with undesirable side effects. This system could also be utilised to screen inhibitors against various PDE4 isoforms in isolation against a low endogenous PDE background *in situ* for isoform-selective inhibitors. BIOCHEM PHARMACOL **57**;12:1375–1382, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cAMP; phosphodiesterase type 4; PDE4 isoforms; CHO cells; PDE4 inhibitors; Sc/Sr binding sites

PDEs† control the level of cyclic nucleotides in the cell by degrading them to inactive metabolites [1, 2], hence playing an essential role in terminating signal transduction processes mediated by the second messengers cAMP and cGMP. Nine PDE families have been described, encoded by distinct genes [1, 3–5], and differing in their substrate preference, sensitivity to endogenous activators and inhibitors, and in their tissue distribution [1–5].

Attention has recently focused on the therapeutic potential of inhibitors of the low K_m cAMP-specific PDE (PDE4) for treatment of asthma and a wide range of inflammatory diseases [6–10]. This is largely due to the tissue distribution of the PDE4 enzymes being expressed in a wide number of cells thought to play a role in allergic and inflammatory responses, including bronchial smooth muscle cells and inflammatory leukocytes [6, 11]. The PDE4 family is encoded by four genes (*A*, *B*, *C*, *D*) [12], each of which encodes multiple forms. The functional significance of the isoforms remains to be established, although there is evidence that N-terminal variants may play a role in cellular localisation [13, 14].

It is currently thought that PDE4 enzymes can exist in two conformations, such that rolipram binds to the catalytic sites of the two conformers with different affinities. One conformer expresses the catalytic site which has a low-affinity for rolipram and is termed the low-affinity rolipram binding site or Sc. The other conformer expresses the catalytic site which has a high-affinity for rolipram and is termed the high-affinity rolipram binding site, HARBS or Sr [15, 16]. This affinity difference is exemplified by *R*-rolipram that binds to Sr with a K_d of 1–5 nM [15–17] but demonstrates up to 100-fold weaker binding to Sc [15–17]. Furthermore, the *R*(−) enantiomer of rolipram demonstrates a 10- to 20-fold selectivity over the *S*(+) enantiomer in binding to Sr, whereas only a 2- to 4-fold selectivity is seen at Sc [15]. These two conformational binding states are exhibited by all four PDE4 enzymes [17]. The rank order of potency of a variety of PDE4 inhibitors for this binding site differs from their rank order of potency for inhibition of cAMP hydrolysis [15]. These results suggest that if the high-affinity rolipram binding site is involved in the regulation of catalytic activity, then not all PDE4 inhibitors act in a purely competitive fashion by binding to the catalytic site on a single form of the enzyme [18]. A truncated version of PDE4A has been

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† *Abbreviations:* PDE, phosphodiesterase; β-AR, β-adrenergic receptor; PKA, protein kinase A; Sr, high-affinity rolipram binding site; Sc, low-affinity catalytic site; CHO cells, Chinese hamster ovary cells; SAFM, serum- and antibiotic-free medium; DPBS(+), Dulbecco's PBS + 0.1% BSA + 0.1% glucose; and cAMP, adenosine 3′,5′-cyclic monophosphate.

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produced lacking 286 amino acids near the N-terminus ($PDE4A_{\Delta Q44\text{-}L329}$, referred to as A_{330}) which, as a consequence, does not express Sr but exhibits identical substrate kinetics to those of the full-length enzyme [17, 19]. Inhibitors such as *R*-rolipram and RS25344, which bind with high affinity to Sr, are significantly less active against the truncated enzyme compared to the full-length enzyme, whereas inhibitors such as CDP840, SB207499, and RP73401 show equal potency against both forms of the enzyme [17].

The majority of cell types contain more than one PDE isoform; thus, in order to characterise the various PDE4 isoforms individually, we have transiently expressed PDE4A, B, C, D, and the truncated A_{330} in CHO L761 cells. This system can also be used for screening inhibitors against specific PDE4 isoforms *in situ*.

MATERIALS AND METHODS

Transient Transfection of Human PDE4 Isoenzymes in CHO Cells

cDNAs encoding PDE4A, B, C, D, and truncated A_{330} as well as the β_2-adrenergic receptor were cloned into the vector pEE7 for transient expression in CHO cells. The PDE cDNA sequences corresponded to the following published sequences: PDE4A (HSPDE4A4B Genbank Acc. No. L20965 [20]), PDE4B (HSPDE4B2A Genbank Acc. No. M97515 [16]), PDE4C (HSPDE4C2 Genbank Acc. No. U88712 [21]), PDE4D (HSPDE4D2 Genbank Acc. No. U50158 [22] and HSPDE4D3A Genbank Acc. No. L20970 [20]), and $PDE4A_{330}$ (ΔQ44-L329 numbered according to Genbank Acc. No. L20965 [19]). PDE4A4, 4C2, and 4D3 were all long forms of the phosphodiesterase 4 enzyme containing upstream conserved regions 1 and 2 (UCR1 and UCR2); PDE4B2 and 4D2 were short forms lacking UCR1, while $PDE4A_{330}$ was a truncated form lacking UCR1 and UCR2. Human genomic DNA was used as a template for the cloning of the β_2-adrenergic receptor by the polymerase chain reaction.

The CHO cells used for transfection were a derivative of CHO-K1, containing engineered *E1A* genes to enhance expression levels, and termed CHO L761 [23]. They were routinely cultured in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1% (v/v) non-essential amino acids, and 1% (v/v) penicillin/streptomycin (all GIBCO BRL). The cells were transfected by the calcium phosphate method or by using lipofectamine (GIBCO BRL). Briefly, in the calcium phosphate method the cells were seeded in 50 mL of medium at 5×10^5 c/mL in a T175 flask (Falcon®, Becton Dickinson) and incubated overnight at 37° and 5% CO_2, after which time the medium was removed and 20 mL fresh medium added to the cells for 3 hr. DNA (200 μg) was added to 1.5 mL of double-strength HEPES balanced salts, then 1.5 mL of 0.25 M calcium phosphate was added dropwise with vigorous shaking. The DNA precipitate was added to the CHO cell culture and incubated for 3 hr. The medium was subsequently removed and 5 mL of 15% glycerol in PBS was added for 1 min at room temperature. The glycerol was then removed by two PBS washes, 50 mL medium added, and the cells cultured for 3 days.

In the lipofectamine method, cells were seeded in 50 mL of complete medium at 2×10^5 c/mL in a T175 flask and incubated overnight. After this time the medium was removed and replaced with 20 mL of SAFM for 30 min. DNA (35 μg) in 2 mL SAFM was added to a further 2 mL SAFM containing 200 μg lipofectamine and allowed to stand at room temperature for 30 min. The SAFM in the flask was then replaced with 16 mL fresh SAFM plus the DNA/lipofectamine mixture (final lipofectamine concentration = 10 μg/mL). After 4–6 hr, 30 mL of complete medium was added, and the cells were fed after 24 hr and cultured for 3 days.

Transient Transfection of Human PDE4 Isoenzymes into COS Cells

cDNAs encoding various PDE4 isoenzymes were cloned into the vector pEE7 and introduced into COS 1 cells by the diethylaminoethyl-dextran method as previously described [19, 21].

Determination of cAMP Elevation in Whole Cells

Three days after transfection the CHO cells were harvested using a non-enzymic dissociation agent (Sigma). The cells were washed three times in ice-cold Dulbecco's PBS + 0.1% BSA + 0.1% glucose (DPBS(+)), resuspended at 2.5×10^6 c/mL in DPBS(+) and stored on ice prior to use. PDE inhibitors were made up as stock solutions of 20 mM in DMSO and diluted 1:500 in DPBS(+) before being added to an equal volume of cell suspension. Cells were preincubated with inhibitors for 10 min at 37° prior to activation. Activation via the β_2-adrenergic receptor was achieved with a 2-min incubation with 0.1 μM isoprenaline at 37°. At the end of activation, samples were immediately microfuged, the supernatant discarded, and the cell pellet resuspended in 400 μL boiling assay diluent (supplied by assay kit manufacturer). The vial was put in a boiling water bath for 10 min and the samples then stored at −20° prior to assay by radioimmunoassay (NEN) or enzyme immunoassay (Amersham) according to the procedure supplied by the manufacturer.

Measurement of PDE Activity

PDE activity was determined by measuring the formation of 5′ AMP by the use of a scintillation proximity assay (Amersham) as described previously [19, 21, 24].

Measurement of Binding at the High-Affinity Rolipram Binding Site (Sr)

Binding at the Sr site was determined in guinea pig brain membranes by the method of Schneider [25], as described previously [19, 21].

Statistical Analysis

To investigate whether statistically significant ($P < 0.05$) relationships existed between elevation of cAMP *in situ* (EC_{50}), inhibition of PDE4 activity, or displacement of [^{3}H]-rolipram from guinea pig brain membranes, data ($\log_{10}$ values) were analysed for linear correlations (r) using the Prism program (GraphPad Software).

Synthesis of PDE4 Inhibitors

All PDE4 inhibitors employed in this study were synthesized in the Department of Medicinal Chemistry at Celltech Therapeutics Ltd. or by Dwight Macdonald at Merck Frosst, Canada. CDP840 (*R*-(+)-4-(2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl) pyridine), RP73401 (*N*-(3,5-dichloro-4-pyridyl)-3-cyclopentoxy-4-methoxy benzamide), RS25344 (1-(3-nitrophenyl)-3-(4-pyridylmethyl)-1,2,3,4-tetrahydro pyrido(2,3-d) pyrimidine-2,4-dione) and *S*-rolipram (*S*-(+)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone) were synthesised at Celltech, while *R*-rolipram (*R*-(−)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidinone) and SB207499 (Tris-(hydroxymethylammonium-4-cyano-4-(3-cyclopentyloxy-4-methoxy-phenyl) cyclohexane carboxylate) were synthesised at Merck Frosst.

RESULTS

Time-course of Isoprenaline-stimulated Elevation of cAMP

The time-course of elevation of cAMP in CHO cells transfected with βAR and PDE4A4 and stimulated with 100 nM isoprenaline in the presence of 10 μM rolipram is shown in Fig. 1A. From this time-course a stimulation time of 2 min was selected for subsequent experiments.

Effect of Isoprenaline and a PDE4 Inhibitor on Mock-transfected CHO Cells

To assess the contribution of any endogenous PDE or β-AR in the CHO cell, a cAMP dose-response to isoprenaline was constructed in the presence and absence of 10 μM rolipram, in a mock-transfected cell. As can be seen in Fig. 1B, no elevation of cAMP was observed over the dose range up to 10 μM isoprenaline, even in the presence of 10 μM rolipram.

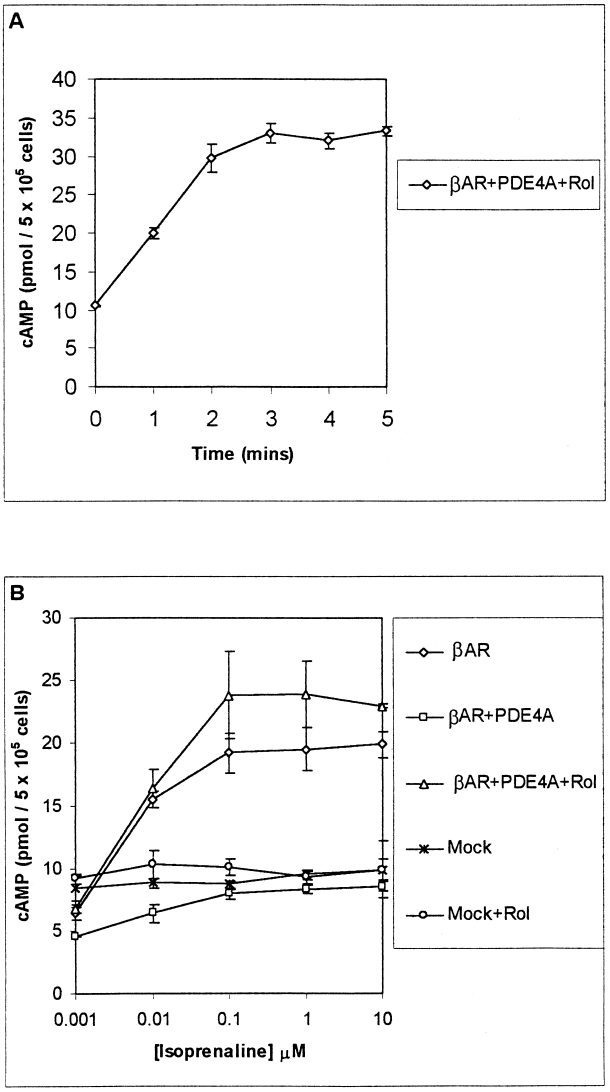

FIG. 1. (A) Time-course of elevation of cAMP in CHO cells transfected with β-adrenergic receptor and PDE4A4. The cells were stimulated with 100 nM isoprenaline in the presence of 10 μM rolipram. (B) Elevation of cAMP in CHO cells was measured as a dose–response to isoprenaline in mock-transfected (Mock) cells and in cells transfected with β-adrenergic receptor (β-AR) alone, or β-AR and PDE4A4, in the presence and absence of 10 μM rolipram (Rol). Data are means ± SEM (N = 3).

Effect of Isoprenaline and a PDE4 Inhibitor on β_2-Adrenergic Receptor and PDE4A-transfected CHO Cells

When β_2-adrenergic receptor-transfected cells were stimulated with isoprenaline, cAMP levels were raised ~3-fold (Fig. 1B). When PDE4A was transfected into the cell as well as β-AR, the cAMP elevation was marginal, demonstrating that the PDE4A was functionally coupled to the cAMP generating system. When this system was stimulated in the presence of 10 μM rolipram, the cAMP was again elevated significantly, in this case ~3.5-fold.

Dose-Response of cAMP Elevation by PDE4 Inhibitors in the Presence of Isoprenaline

CHO cells were transiently transfected with the β_2-adrenergic receptor and a PDE4 enzyme. The PDE4 isoforms that were transfected were 4A4, 4B2, 4C2, 4D2, 4D3, and $4A_{330}$. In each case the elevation of cAMP was measured as a dose–response to a panel of PDE4 inhibitors in the presence of a suboptimal concentration of 100 nM isoprenaline. The results are shown in Fig. 2. The inhibitor profiles were similar in each case, with the exception of the truncated $PDE4A_{330}$. RS25344, which was the most potent elevator of cAMP in cells transfected with PDE4A, 4B, 4C, and 4D, was virtually inactive when $PDE4A_{330}$ was transfected into the CHO cells. The same was true of other inhibitors that were more potent at Sr than Sc, such as rolipram. However, CDP840, RP73401, and SB207499 were able to elevate cAMP equivalently whether the cells were transfected with full-length PDE4A or the truncated $PDE4A_{330}$. In addition, RS25344 appeared to give a biphasic dose–response curve with PDE4D3, and to some extent with PDE4D2.

Correlations

The ability of the various inhibitors to elevate cAMP by inhibition of the various isoforms *in situ* was correlated with their ability to inhibit either at the Sr site of guinea pig brain membranes or at the Sc site of $PDE4A_{330}$ (ex-COS) *in vitro*, and the results are shown in Table 1. The results show that elevation of cAMP by inhibition of PDE4A4, 4B2, 4C2, 4D2, and 4D3 all correlate with inhibition of the Sr site and do not correlate with inhibition of the Sc site of $PDE4A_{330}$, demonstrating that they exhibit high-affinity binding when expressed in the CHO cell. The converse is true of $PDE4A_{330}$: when expressed in the CHO cell its inhibition *in situ* correlates with inhibition of $PDE4A_{330}$ *in vitro* and not with inhibition of the Sr site, indicating that the $PDE4A_{330}$ adopts a low-affinity conformation *in situ*.

These *in situ* correlations were extended to include PDE4A4 (ex-COS) and PDE4C2 (ex-COS) (Table 1). It can be seen that the correlation profile obtained with PDE4A4 mirrors that obtained with $PDE4A_{330}$ (i.e. low affinity); similarly, the correlation profile obtained with PDE4C2 mirrors that obtained with inhibition of the high-affinity Sr binding site.

A further correlation ($r = 0.995$, $P = 0.0001$) was observed when a range of PDE4 inhibitors was used to determine IC_{50} values on PDE4A4 derived from CHO cells and COS cells (see Fig. 3).

The Effect of Stimulation of PDE4A4 In Situ *on Specific Activity and Inhibitor Potency (ex-CHO)*

PDE4A4 demonstrated an ~3-fold increase in specific activity upon stimulation *in situ* in CHO cells (Table 2); however, stimulation did not significantly affect the IC_{50} values determined for a range of PDE4 inhibitors (Table 3).

DISCUSSION

We have described the transient expression of various PDE4 enzymes in the CHO cell, enabling the study of individual PDE4 isoforms, in isolation, against a low endogenous PDE background. A 2-min stimulation time was selected for use in subsequent experiments from the time-course of isoprenaline-stimulated cAMP elevation shown in Fig. 1A. No elevation of cAMP was observed in a mock-transfected CHO cell stimulated with isoprenaline in the presence of 10 μM rolipram (Fig. 1B). This indicates the absence of endogenous β-adrenergic receptors (β-AR) in CHO cells, which is in agreement with previously published work [26]. Upon transfection of β-AR into the CHO cell, an isoprenaline-stimulated elevation of cAMP was obtained, indicating successful transfection and coupling to adenylyl cyclase (Fig. 1B). When PDE4A was co-transfected with β-AR, the stimulated elevation of cAMP was dramatically reduced, indicating that the PDE was functionally coupled into the cAMP generating system. This transfected PDE4A could be fully inhibited by 10 μM rolipram, and as the cAMP level attained was slightly higher than with β-AR transfection alone, it appeared that a low level of an endogenous rolipram-sensitive PDE was being revealed. When the PDE activity was measured in homogenised CHO cells, the transfected PDE represented >97.7% of the total measurable PDE. From the *in situ* result, it would appear that the majority of this transfected PDE is functionally coupled.

Dose–response curves of cAMP elevation were constructed for a range of PDE4 inhibitors tested on CHO cells expressing β-AR and various PDE4 enzymes (Fig. 2). The PDE4 isoforms expressed were 4A4, 4B2, 4C2, 4D2, 4D3, and $4A_{330}$. The cells were stimulated with 100 nM isoprenaline, a suboptimal concentration which alone gave no increase in cAMP, but in the presence of a PDE inhibitor an elevation of cAMP was evident. The dose–response curves were very similar in each case, with the exception of $PDE4A_{330}$. The most obvious difference was with RS25344, which was the most potent elevator of cAMP against each PDE4 isoform, but was virtually inactive against $PDE4A_{330}$. *R*-rolipram was also relatively weaker against the truncated enzyme. A further difference was that the dose–response curves for elevation of cAMP in the presence of RS25344 appeared to be biphasic with PDE4D2 and PDE4D3. This may be related to the ~100-fold shift in potency reported for RS25344 when PDE4D3 is phosphorylated by PKA [27]. PDE4D2 does not contain the PKA consensus sites at Ser 13 and Ser 54, but the PDE4D3 double mutant (Ser 13 and Ser 54 ⇒ Ala) still undergoes PKA-dependent phosphorylation, albeit at a lower level [28]. The impact of this phosphorylation, or indeed a PKA-independent phosphorylation, which may also occur in PDE4D2, on inhibitor potency is unknown.

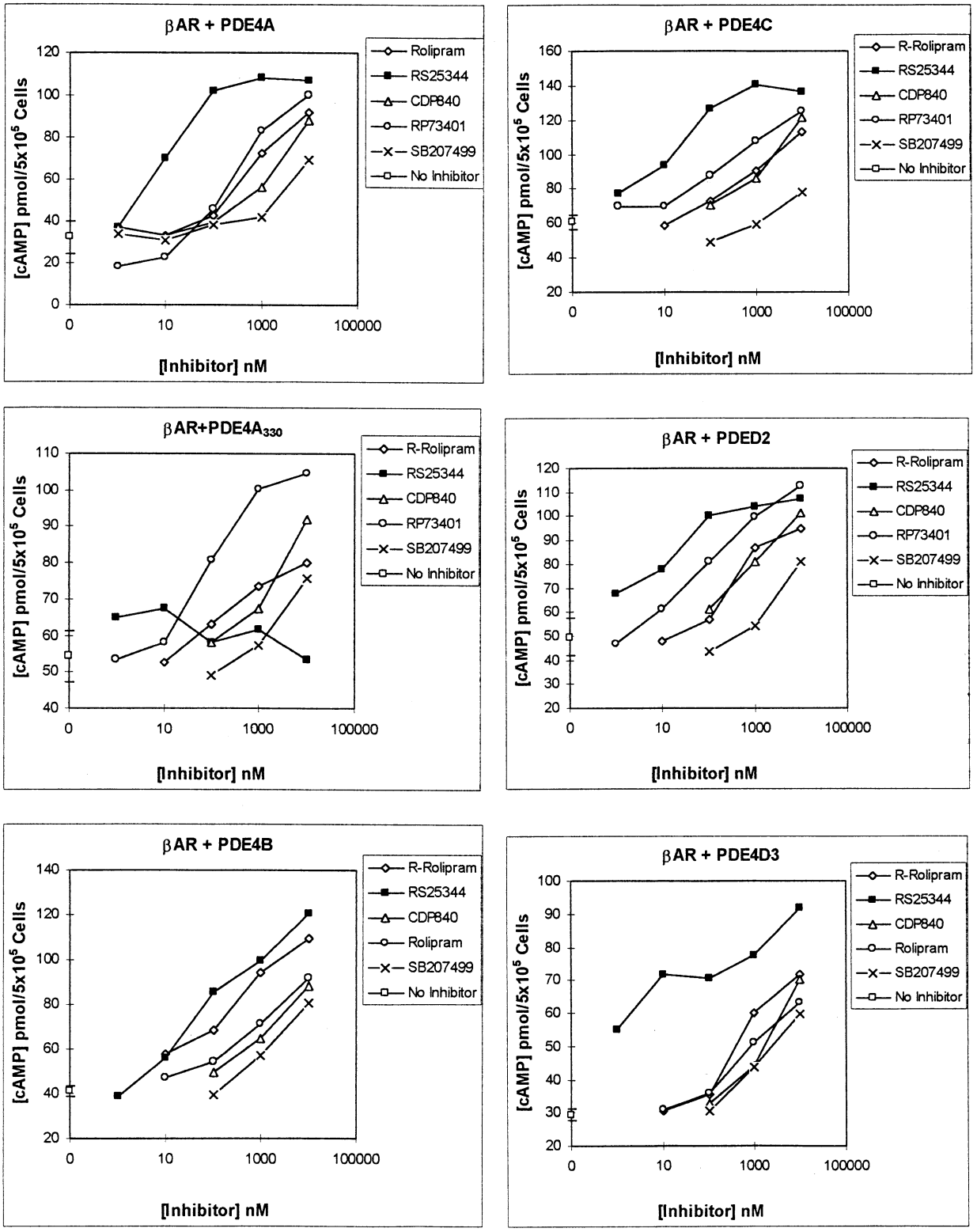

FIG. 2. Elevation of cAMP in CHO cells transfected with β-adrenergic receptor and various PDE4 isoforms. The cells were stimulated with 100 nM isoprenaline in the presence of a concentration range of various PDE4 inhibitors. These are representative results from transfections that were repeated at least three times. Error bars have been omitted for clarity.

The EC_{50} values derived from the dose–response curves of cAMP elevation for the PDE4 inhibitors for each PDE isoform were analysed for a correlation with the inhibition of [^{3}H]-rolipram binding to the Sr site of guinea pig brain membranes, representing a high-affinity binding site, and with the inhibition of the catalytic activity (Sc) of the truncated $PDE4A_{330}$ (ex-COS), representing a low-affinity binding site. It is clear from the results, shown in Table 1,

TABLE 1. Correlation of the elevation of cAMP *in situ* in CHO cells (EC_{50}) with the inhibition of PDE4 activity *in vitro* (IC_{50}) or inhibition of the rolipram high affinity binding site (Sr) in guinea pig brain membranes

		In vitro			
		Sr (GPbm)	$PDE4A_{330}$ (ex-COS)	PDE4A4 (ex-COS)	PDE4C2 (ex-COS)
In situ (CHO)	PDE4A4	0.898 ($P = 0.001$)	−0.199 (NS)	0.429 (NS)	0.910 ($P = 0.0007$)
	PDE4B2	0.788 ($P = 0.012$)	−0.530 (NS)	0.166 (NS)	0.856 ($P = 0.003$)
	PDE4C2	0.717 ($P = 0.020$)	−0.175 (NS)	0.381 (NS)	0.935 ($P < 0.0001$)
	PDE4D2	0.728 ($P = 0.041$)	−0.365 (NS)	0.319 (NS)	0.945 ($P = 0.0004$)
	PDE4D3	0.705 ($P = 0.034$)	−0.305 (NS)	0.341 (NS)	0.860 ($P = 0.003$)
	$PDE4A_{330}$	0.260 (NS)	0.810 ($P = 0.027$)	0.808 ($P = 0.015$)	0.343 (NS)

The values in the table are the linear correlations of the $\log_{10}$ data (r) and the statistical significance (P) derived using the Prism computer program. GPbm, guinea pig brain membranes; NS, not significant.

that inhibition of PDE4A4, 4B2, 4C2, 4D2, and 4D3, *in situ*, all correlate with binding at the high-affinity [^{3}H]-rolipram binding site in guinea pig brain membranes, whereas inhibition of $PDE4A_{330}$ *in situ* does not.

In addition, the converse relationship is also evident. Elevation of cAMP as a result of inhibition of $PDE4A_{330}$ in the CHO cell correlates with inhibition of the low-affinity catalytic activity of the isolated $PDE4A_{330}$, whereas inhibition of all the other PDE4 isoforms, *in situ*, does not correlate with inhibition of the low-affinity Sc binding site. Thus, it is clear that all the isoforms of PDE4 expressed transiently in the CHO cell, with the exception of the truncated $PDE4A_{330}$, adopt a high-affinity conformation.

When further *in vitro* data were analysed for correlations with the data generated *in situ*, it was clear that the profile of correlations for inhibition of PDE4A (ex-COS) most closely resembled $PDE4A_{330}$ (ex-COS) (Table 1). These two forms of the PDE4 enzyme only gave correlations with the $PDE4A_{330}$ *in situ* data, leading to the conclusion that PDE4A (ex-COS) adopts a low-affinity conformation. A similar correlation profile was also obtained with PDE4B and PDE4D (ex-COS), demonstrating that they too adopt a low-affinity conformation *in vitro* (data not shown). Conversely, the profile of correlations for PDE4C (ex-COS) was very similar to that for binding at the Sr site of guinea pig brain membranes, in that neither correlated with $PDE4A_{330}$ *in situ*, but instead correlated with all the other isoforms expressed in the CHO cells, implying that a high-affinity conformation for PDE4C2 (ex-COS) is retained *in vitro*. These data are in agreement with previous reports [17, 21].

As the *in vitro* data were largely generated from PDE4 enzymes expressed in COS cells, some experiments were performed with PDE4A expressed in CHO cells to compare different cellular sources of enzyme. The *in vitro* inhibition of PDE4A (ex-CHO) by a range of PDE4 inhibitors correlated extremely well ($r = 0.995$) with *in vitro* inhibition of PDE4A (ex-COS) (Fig. 3), implying that

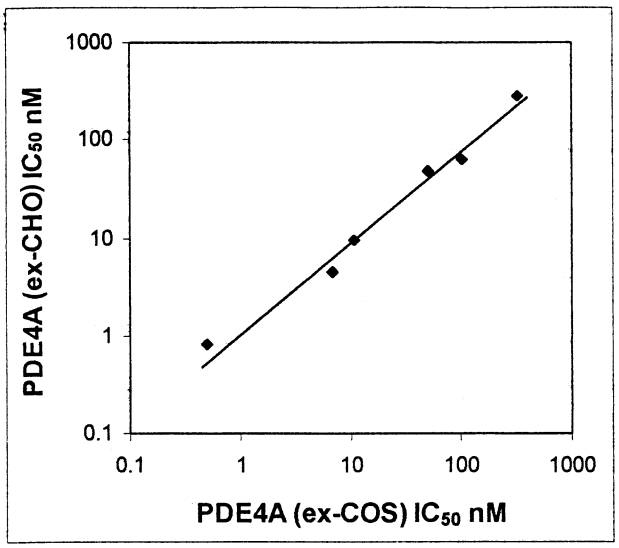

FIG. 3. Correlation of the *in vitro* inhibition of PDE4A4 ex-CHO and ex-COS by a range of PDE4 inhibitors (*R*-rolipram, *S*-rolipram, CDP840, RS25344, SB207499, and RP73401). The data ($\log_{10}$ values) were analysed to derive a Pearson's linear correlation of $r = 0.995$, which was highly significant ($P = 0.0001$). This analysis was carried out using the Prism computer program by GraphPad software.

TABLE 2. The effect of stimulation *in situ* on the specific activity *in vitro* of PDE4A4 ex-CHO

	Specific activity (pmol cAMP/min/mg)
Unstimulated	82.6 ± 21.4
Stimulated	239 ± 61.2

Cells were stimulated with 0.1 μM isoprenaline for 2 min. Data are means ± SD of three separate assays, each carried out in duplicate, on a single transfection.

TABLE 3. The effect of stimulation *in situ* on the IC_{50} of PDE4 inhibitors *in vitro* on PDE4A4 ex-CHO

	IC_{50} (nM)	
	Unstimulated	**Stimulated**
R-rolipram	62.3 ± 5.1	68.3 ± 5.5
S-rolipram	211 ± 6.5	263 ± 15.6
CDP840	5.3 ± 0.5	4.6 ± 0.6
RS25344	14.6 ± 4.6	11.6 ± 2.1
SB207499	61.3 ± 14.5	58.3 ± 11.7
RP73401	1.4 ± 0.4	1.0 ± 0.3

Cells were stimulated with 0.1 μM isoprenaline for 2 min. Data are means ± SD of three separate assays, each carried out in duplicate, on a single transfection.

PDE4A expressed in both systems gives a low-affinity enzyme *in vitro*. This was confirmed for PDE4A (ex-CHO) by its correlation with $PDE4A_{330}$ (ex-COS) ($r = 0.834$), its lack of correlation with binding at the high-affinity Sr site ($r = 0.482$), and its lack of correlation with inhibition of PDE4A *in situ* in the CHO cell ($r = 0.491$), which exists in a high-affinity conformation (data not shown). In addition, preliminary data demonstrate further correlations between the *in vitro* inhibition of PDE4B2 ($r = 0.858$), PDE4C2 ($r = 0.998$), and PDE4D3 ($r = 0.847$) expressed in COS and CHO cells, all reaching statistical significance (data not shown).

In thinking about why all the non-truncated PDE4 enzymes expressed in the CHO cell adopted a high-affinity conformation, we considered whether stimulation with isoprenaline leading to the elevation of cAMP and subsequent activation of PKA could have caused phosphorylation of the PDE4 enzymes. To address this question, we first looked to see if stimulation of PDE4A4 in the CHO cell altered the specific activity *in vitro*. As can be seen in Table 2, the specific activity of PDE4A4 increased ~3-fold upon stimulation. Phosphorylation of isoforms of most PDE classes has been reported, and in some cases this has been associated with changes in hydrolytic activity [29]. Activation by phosphorylation has been observed in human PDE4D3 [27] and is proposed to be due to phosphorylation of Ser 54 [28, 30]. This phosphorylation causes a dramatic increase in the potency of rolipram [28] and two nitroquazone analogues, including RS25344 [27], and is believed to be due to an increase in the proportion of the high-affinity conformer. The same consensus sequence around Ser 54 is present in certain splice variants derived from *PDE4A*, *PDE4B* and *PDE4C* genes [20], but is lacking in two other PDE4D splice variants, PDE4D1 and PDE4D2, that are truncated at the N-terminus and are not good substrates for, nor activated by, PKA [31]. However, in contrast to PDE4D3, stimulation of PDE4A4 *in situ* in the CHO cell does not significantly alter the IC_{50} values determined for a range of PDE4 inhibitors *in vitro*, including *R*-rolipram and RS25344 (see Table 3). In addition, *all* the PDE4 isoforms expressed in the CHO cell (with the exception of the truncated $PDE4A_{330}$) adopt the high-affinity conformation, while only three of the isoforms contain the consensus Ser 54 (PDE4A4, PDE4C2, and PDE4D3) and two isoforms do not (PDE4B2 and PDE4D2). One possible explanation for this is that there are sites in PDE4D3, in addition to Ser 13 and Ser 54, that are phosphorylated by PKA, or indeed another kinase. The double Ser ⇒ Ala mutant is still PKA-phosphorylated, albeit at a lower level, and has substantial activity [28]. These site(s) may become phosphorylated upon stimulation, leading to a conformational change or allowing the PDE4 enzymes to interact with regulatory proteins, such as membrane or cytoskeletal proteins. These putative phosphorylations could also be occurring prestimulation, as the basal phosphorylation state of the PDEs in the cell is unknown. It is possible that a completely de-phosphorylated PDE4 *in situ*, if it could be achieved, would exhibit a low-affinity conformation. However, the truncated $PDE4A_{330}$ does exist as a low-affinity conformer when expressed in the CHO cell. This gives the opportunity of using this system to screen and optimize inhibitors against a low-affinity conformation of PDE4 *in situ* and use a high-affinity conformation of PDE4 as a counterscreen, as inhibitor activity against this conformer has been linked with undesirable side effects including emesis [32], gastric acid secretion [18], and psychotropic activity [33]. The system could also be utilised to screen inhibitors against the various PDE4 isoforms in isolation against a low endogenous PDE background *in situ* to look for isoform-selective inhibitors.

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