BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **241,** 175–180 (1997)
ARTICLE NO. RC977542

Zinc Dependent Activation of cAMP-Specific Phosphodiesterase (PDE4A)[1]

M. David Percival, Brian Yeh, and Jean-Pierre Falgueyret
Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Kirkland, Quebec, Canada

Received September 23, 1997

Cyclic nucleotide phosphodiesterases (PDE), including PDE4A, contain two consensus sequences ($HX_3HX_{24-26}E$) which have been associated with Zn^{2+} binding and activation with other proteins. This study shows that Zn^{2+} activates purified recombinant human PDE4A with an EC_{50} of <1 μM. The EC_{50} for Mg^{2+}, the generally accepted activating metal ion, is $\approx$100 μM. Zn^{2+} concentrations higher than 5 μM are inhibitory. Mn^{2+}, Co^{2+} and Ni^{2+} also activate PDE4A with EC_{50} values of approximately 2, 3 and 10 μM, respectively. PDE4A binds $^{65}Zn^{2+}$ with a K_d of 0.4 μM and $\approx$1:1 stoichiometry. Titrations of PDE4A inhibition with Mg^{2+} and Zn^{2+} as activating metal ions showed that the competitive inhibitors R-Rolipram, CDP-840, RS-14203 and KF18280 are shifted at least 10-fold to lower potency in the presence of Zn^{2+}. The effect is likely at the site of inhibitor binding as the K_m for cAMP in the presence of Mg^{2+} and Zn^{2+} is similar (1-3 μM). The K_d of [^{3}H]-R-Rolipram for PDE4A was increased at least 30-fold from 3 nM (with Mg^{2+}) by the presence of Zn^{2+}. The high affinity of Zn^{2+} for PDE4A indicates that this metal may play a role in the regulation of PDE4A activity.

Cyclic nucleotide phosphodiesterases (PDE) catalyze the hydrolysis of cAMP or cGMP to their respective phosphate monoesters and, as such, control the intracellular concentrations of these important second messengers. Seven gene families of PDEs have been identified and are distinguished on the basis of their substrate specificities, regulation, inhibitor sensitivities and tissue distribution (1-3). PDE4, which is specific for cAMP, has a low substrate K_m and is sensitive to inhibition by Rolipram (4) is of interest as a target for anti-asthmatic and anti-inflammatory therapy (5). The PDE4 family contains four gene products (PDE4A-D), the diversity of which is further increased by differential mRNA splicing and post translational modification (6). All PDEs sequenced to date contain a conserved sequence of approximately 270 residues that likely contains the catalytic domain (2). Within this conserved region exists two sequences ($HX_3HX_{24-26}E$) that have a high degree of homology to the Zn^{2+}-binding sequence of thermolysin (7). Bovine lung cGMP-binding, cGMP-specific PDE (PDE5) and ROS-PDE (PDE6) both bind and are activated by Zn^{2+} (7, 8), as is leukotriene A_4 hydrolase (9), which also contains the same Zn^{2+}-binding consensus sequence. From the sequence homologies of the PDE families and the fact that all PDEs require a divalent metal ion for activity (assays are generally performed in the presence of 1-10 mM Mg^{2+}), it may be expected that a similar Zn^{2+} dependency would be exhibited by PDE4. However, cGMP-inhibited PDE (PDE3) and PDE4D1 are not activated by Zn^{2+}, although PDE3 is inhibited by low concentrations of Zn^{2+} (10, 11). This report describes the metal ion dependency of PDE4A activation and inhibition.

MATERIALS AND METHODS

Purification buffers were of the highest available purity and all assay buffers and cAMP were passed through a chelex membrane (BioRad). Recombinant human PDE4A was expressed in a baculovirus expression system (12). Sf9 cells (1 L) were grown to a concentration of 0.5×10^6 cells/mL in Grace's insect cells medium (Gibco-BRL) supplemented with 10% heat inactivated fetal bovine serum (Gibco-BRL) in the presence of 50 mg per liter of gentamicine. Cells were infected at an MOI of 5 to 10 and collected 48 hours after the infection (viability $>$ 80%). The cells were centrifuged, resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM DTT, 10 μg/ml pepstatin A, 1 mM BAEE, 1 mM benzamidine and protease inhibitor cocktail (Complete, Boehringer)) and disrupted by sonication. The lysate was centrifuged (15 min 10,000 $\times$ g, 4°C) and the supernatant

[1] A preliminary account of this work was presented at the American Thoracic Society Annual Meeting, San Francisco, May 18–22, 1997.

Abbreviations: BAEE; N-benzoyl-L-arginine ethyl ester, ICP; inductively coupled plasma, MOI; multiplicity of infection, PDE; Cyclic nucleotide phosphodiesterase, PKA; protein kinase A, ROS; Rod outer segment, SPA; scintillation proximity assay.

0006-291X/97 $25.00

recentrifuged for 1 hour (100,000 × g). The supernatant was applied to a Q-sepharose column (Pharmacia, 1.6 × 10 cm) equilibrated with buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 mM DTT) prior to elution with a linear gradient (0 to 1 M KCl) over 10 column volumes. Active fractions were pooled and applied to a Cibacron blue agarose gel column (Sigma, 1.0 × 10 cm) equilibrated with buffer A. After washing with 10 volumes of buffer A containing 1 M KCl, the PDE4A was eluted with 50 mM Tris pH 10.6, 1 M KCl, 2 mM adenosine. The fractions were neutralized to pH 8.0 with 1 M Tris-HCl pH 7.0. Active fractions were pooled and the protein concentrated and dialyzed (Buffer A) by diafiltration (Amicon, YM10 membrane). As a final step the enzyme was applied to a POROS-Q column (0.46 × 5 mm, Boehringer) equilibrated with buffer A and eluted with a linear gradient of KCl (0 to 1 M) over 10 column volumes. PDE4 is highly susceptible to proteolysis, necessitating the presence of EDTA during the purification (14). The purified protein was concentrated to 0.1-0.3 mg/mL (Millipore, Ultrafree-30) and dialyzed (1000:1v/v) for 6 h against two changes of 12 mM Chelex-treated HEPES, pH 7.0, 100 mM NaCl to ensure the removal of EDTA and adventitious metal ions. The final POROS column step was also performed without EDTA to ensure that trace amounts did not interfere in the binding assays in which relatively large volumes of enzyme was used. No differences in activity or behavior in the binding assays were observed between enzyme purified (POROS column) in the absence or presence of EDTA. Protein concentrations were measured by Coomassie blue binding (BioRad) using BSA standard.

Metal ion analysis by ICP was performed by The Chemical Analysis Laboratory (University of Georgia, Athens, GA). Cibacron blue column-purified PDE4A was dialyzed against two changes of chelex-treated 5 mM HEPES, pH 7.0 for 24 h. No loss of activity was observed over this time. The protein was concentrated on a Centricon-30 microconcentrator (Amicon).

PDE activity was measured at room temperature by a scintillation proximity assay (Amersham) in 200 μL 50 mM HEPES, pH 7.0, containing 0.1 μM [^{3}H]cAMP (0.2 μCi), 1 mM EDTA and 10 mM $MgCl_2$ plus approximately 15 ng PDE4A. The reaction time was 10 min and the reaction was stopped by the addition of 65 μL SPA beads. The amount of product formed was always less than 10%. Inhibitors were dissolved in DMSO and added to the reaction buffer such that the DMSO concentration was 1% (v/v). The reaction was initiated with enzyme.

$^{65}Zn^{2+}$-binding to PDE4A was determined in a filtration binding assay (7) in which PDE4A was added to 50 μL of a mixture containing 10 mM HEPES, pH 7.0, 100 mM NaCl and 0-3 μM $^{65}Zn^{2+}$ (262 cpm/pmol). After 15 min, 1 mL of ice cold 5 mM HEPES, pH 7.0, 50 mM NaCl was added and the total volume filtered under vacuum on a HAWP membrane (Millipore) which was previously wetted with cold buffer. The filter was rapidly washed with 2 mL cold buffer before counting.

[^{3}H]-(R)-Rolipram binding to PDE4A was determined in a rapid filtration binding assay (13) in which PDE4A (0.84 μg, 8.4 pmol) was incubated for 10 min in 100 μL 50 mM HEPES, pH 7.0, 1 μM EDTA, 5 mM Mg^{2+} or 4 μM Zn^{2+} with 0.1-100 nM [^{3}H]-(R)-Rolipram (0.022 μCi/pmol), prior to rapid filtration (Tomtec Harvester96) through GF/B filters presoaked in 1% polyethyleneimine. The filters were washed with ice cold 50 mM Tris, pH 7.5 and then counted.

RESULTS

Expression and Purification of PDE4A

Recombinant full length PDE4A was expressed in a baculovirus-Sf9 cell system (12) and purified to greater than 90% homogeneity by a scheme involving Q-sepharose, Cibacron blue and POROS Q chromatography (Table 1). The final POROS column step gave a significant increase in specific activity but yielded low recoveries (Table 1). It appears that full length PDE4 is stable and readily manipulated in an impure state, but undergoes aggregation (15) when highly purified.

Activation of PDE4A by Divalent Metal Ions

The concentration dependence of a variety of divalent metal ions on PDE4A activity was determined in the presence of buffer containing 1.0 μM EDTA. The results (Fig. 1) show that in the absence of added metal ions insignificant activity was detected. If EDTA was omitted variable activities (0.05-0.1 μmol/min/mg), considerably higher than background were observed, indicating that very low levels of adventitious metal ions are capable of supporting enzyme catalysis. Of the metals tested, Zn^{2+} was effective at stimulating PDE4 activity at the lowest concentration ($EC_{50}\approx0.5$ μM), followed by Mn^{2+} ($EC_{50}\approx2$ μM), Co^{2+} ($EC_{50}\approx3$ μM), Ni^{2+} ($EC_{50}\approx10$ μM) and Mg^{2+} ($EC_{50}\approx100$ μM). Ca^{2+}, Cu^{2+} and VO^{2+} did not stimulate activity (data not shown). These results parallel almost exactly those observed with PDE5 (7). Similarly, PDE4D and PDE3 are also activated by Mn^{2+}, Co^{2+} and Mg^{2+}, but not by Zn^{2+} (10, 11). The effect of Zn^{2+} was maximal at about 1-3 μM Zn^{2+}, higher concentrations resulted in inhibition of PDE4A activity. The inhibition by Zn^{2+} does not appear to be competitive with Mg^{2+} or Co^{2+}. At 100 μM Zn^{2+}, no increase in activity was observed when Mg^{2+} or Co^{2+}

TABLE 1

Purification of PDE4A

Step	Protein (mg)	Activity[a] (nmol/min)	Specific activity[a] (nmol/min/mg)	Purification (fold)	Yield (%)
S100	269	105	0.39	—	—
Q-Sepharose	158	109	0.69	1.8	100
Cibacron blue	4.7	42	8.85	23	40
Diafiltration	4	48	12.16	31	45
POROS-Q	0.26	11.6	44.8	115	11

[a] Activity was measured in the presence of 0.1 μM cAMP.

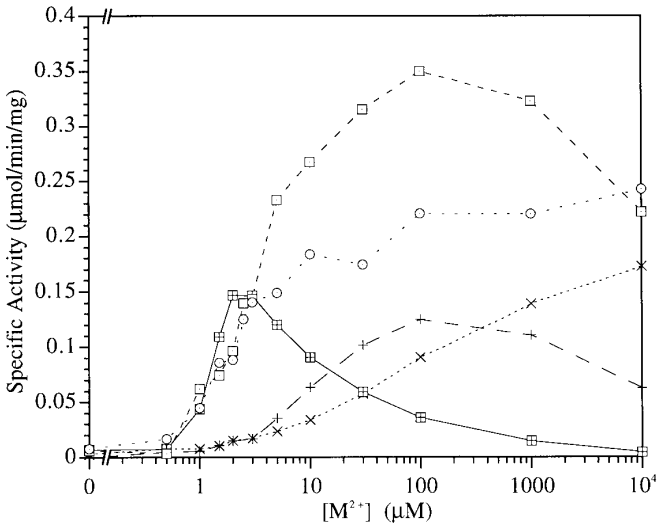

FIG. 1. Effect of Metal Ions on PDE4A Activity. PDE activity was measured by SPA (Materials and Methods) in the presence of Mg^{2+} (X), Zn^{2+} (ii/home2/milesgrsym/8point/gs91,1(3,0)), Co^{2+} (⊡), Mn^{2+} (⊙) and Ni^{2+} (+). The metal free 50 mM HEPES, pH 7.0 buffer also contained 2 μM cAMP, 1.0 μM EDTA and 15 ng purified PDE4A. The results are the average of duplicate determinations.

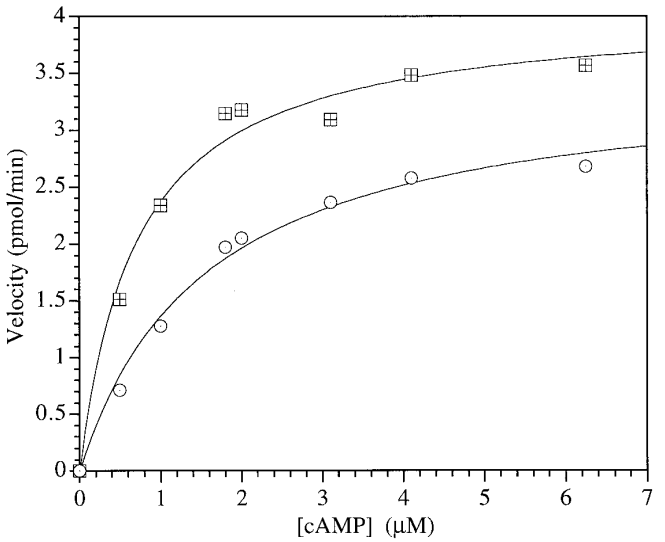

FIG. 2. Determination of cAMP K_m in the presence of Mg^{2+} and Zn^{2+}. PDE activity was measured by SPA (Materials and Methods) in the presence of 10 mM Mg^{2+} (ii/home2/milesgrsym/8point/gs91,1(3,0)) and 3 μM Zn^{2+} (⊙). The metal free 50 mM HEPES, pH 7.0 buffer contained 2 μM cAMP, 1.0 μM EDTA and 9 ng purified PDE4A. The results are the average of duplicate determinations.

concentrations were increased from 10 μM to 10 mM. Inhibition by Zn^{2+} has also been observed with PDE5 (7), PDE3 (10), leukotriene A_4 hydrolase (16) and thermolysin (17), although in the case of PDE3, the inhibition is apparently competitive with the activating metal ion, Mn^{2+}. For thermolysin, the inhibitory Zn^{2+} binds at a second distinct site (17).

Substrate K_m in the Presence of Mg^{2+} and Zn^{2+}

The substrate K_m for cAMP hydrolysis was measured at optimal Mg^{2+} (10 mM) and Zn^{2+} (2 μM) concentrations (Fig. 2). The K_m values obtained for Mg^{2+} (0.8 ± 0.2 μM) and Zn^{2+} (2.1 ± 0.4 μM) are within a factor of 2-3 and are close (for Mg^{2+}) to reported literature values (14, 18). The V_{max} for PDE4A with Mg^{2+} (0.45 μmol/min/mg) is considerably higher than recently reported (18) for PDE4A expressed by baculovirus-Sf9 (0.057 μmol/min/mg), but is similar (0.8 μmol/min/mg) to PDE4A expressed in yeast (14) and PDE4D1 (0.318 μmol/min/mg) expressed in E. coli (11).

$^{65}Zn^{2+}$ Binding to PDE4A

Binding of $^{65}Zn^{2+}$ to PDE4A was determined in a non-equilibrium filtration binding assay. The saturation curve obtained (Fig. 3) gave a K_d of 0.4 ± 0.1 μM Zn^{2+} and a B_{max} of 1.0 ± 0.1 mol Zn^{2+}/mol PDE4A. Maximal binding was obtained after incubation of enzyme and $^{65}Zn^{2+}$ for the minimum time tested (15 min) and was stable for up to 4 h. Heat denaturation of PDE4A reduced $^{65}Zn^{2+}$ binding by greater than 85%. $^{65}Zn^{2+}$ binding (at 1 μM) was completely ablated by co-incubation of PDE4A with 10 μM cold Zn^{2+} or 10 μM EDTA, but was only reduced 10% by the presence of 50 mM Mg^{2+}.

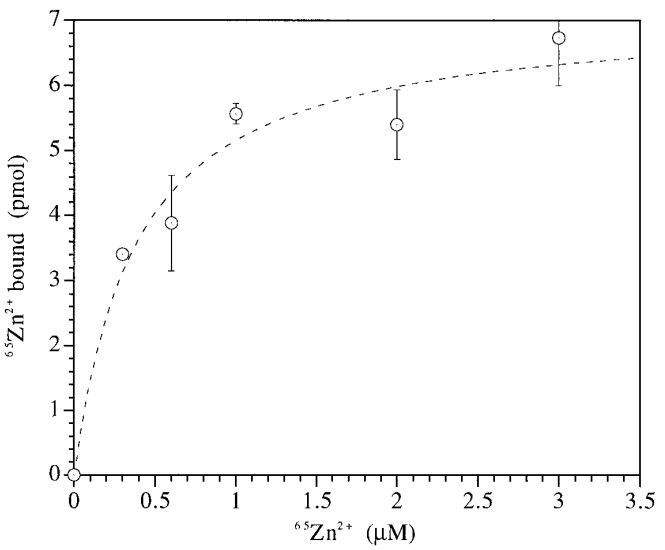

FIG. 3. Concentration Dependence of $^{65}Zn^{2+}$ Binding. $^{65}Zn^{2+}$ binding to PDE4A (0.71 μg, 7.1 pmol) was measured in a filtration assay as described (Materials and Methods). The data points are the average of duplicate determinations and the error bars represent the range. The data was fitted to the standard single binding site equation which gave a K_d of 0.4 ± 0.1 μM and B_{max} of 1.0 ± 0.1 mol Zn^{2+}/mol PDE4A. Less pure preparations of PDE4A gave similar K_d values but lower B_{max} values (0.5 - 1 mol Zn^{2+}/mol PDE4A).

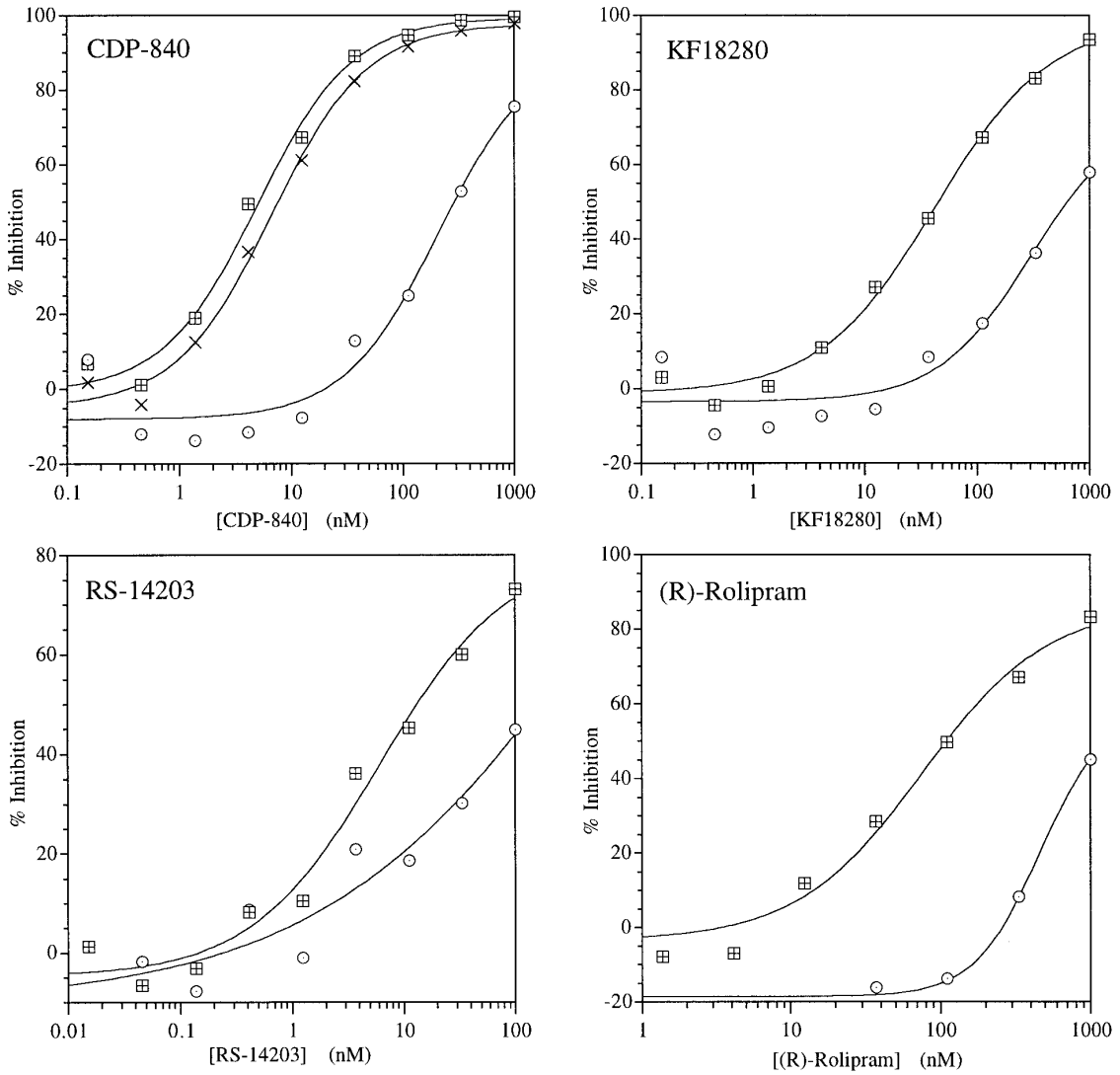

FIG. 4. Effect of Mg^{2+} and Zn^{2+} on Inhibitor Sensitivity of PDE4A. Titrations of PDE4A inhibitors were measured in the presence of 10 mM Mg^{2+} (ii/home2/milesgrsym/8point/gs91,1(3,0)) and 3 μM Zn^{2+} (⊙) as described (Materials and Methods). The titration of CDP-840 was also performed in the presence of 10 mM Mg^{2+} and 3 μM Zn^{2+} together (X). The metal free 50 mM HEPES, pH 7.0 buffer also contained 0.1 μM cAMP, 1.0 μM EDTA and 9 ng purified PDE4A. The results are the average of duplicate determinations and are representative of 2 separate experiments. The IC_{50} values were calculated using a four parameter least squares regression analysis and are as follows; (Mg^{2+}, Zn^{2+}), CDP-840 (5 nM, 240 nM); R-Rolipram (110 nM, >1000 nM), KF18280 (45 nM, 650 nM); RS-14203 (10 nM, >100 nM).

A half time for the dissociation of $^{65}Zn^{2+}$ of approximately 30 s was measured by treating the preformed $^{65}Zn^{2+}$-PDE4A complex (1 μM $^{65}Zn^{2+}$) with an excess of EDTA (10 μM) followed by rapid filtration at timed intervals.

The results obtained for $^{65}Zn^{2+}$ binding with PDE4A are very similar to those obtained with PDE5 (7). In the latter case the K_d for Zn^{2+} was also approximately 0.5 μM, but a stoichiometry of $\approx$ 3 mol $^{65}Zn^{2+}$/mol enzyme was obtained. The lower stoichiometry (1.0 mol $^{65}Zn^{2+}$/mol enzyme) obtained in this study may result from the presence of inactive PDE4A, or may reflect differences in the metal ion binding of the two PDE isozymes. It is not likely due to prior occupation of the binding site(s) since the enzyme was purified in the presence of EDTA which was shown to rapidly remove Zn^{2+}. Metal ion analysis of purified PDE4A showed insignificant amounts of Zn^{2+} or other metal ions.

PDE4A Inhibitor Sensitivity and [^{3}H]-(R)-Rolipram Binding

An increase in IC_{50} value of at least 10-fold was observed for each inhibitor tested when Mg^{2+} was re-

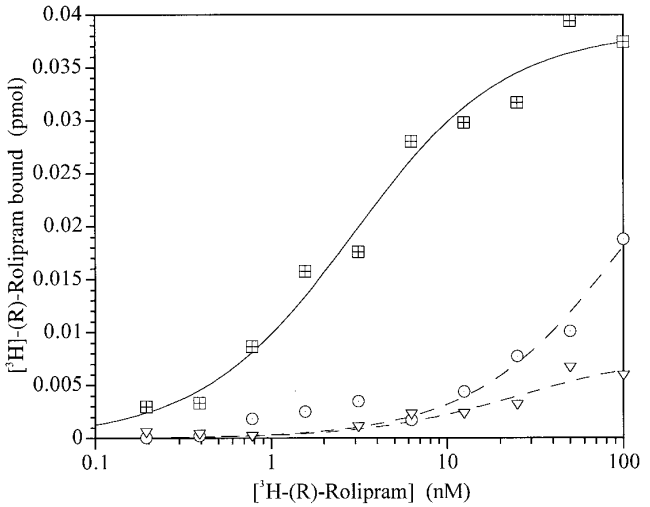

FIG. 5. Effect of Mg^{2+} and Zn^{2+} on [^{3}H]-(R)-Rolipram Binding to PDE4A. [^{3}H]-(R)-Rolipram binding to PDE4A (8.4 pmol) was measured in the presence of 5 mM Mg^{2+} (ii/home2/milesgrsym/8point/gs91,1(3,0)), 4 μM Zn^{2+} (⊙) and no added metal (▽) in a filtration assay as described (Materials and Methods). The data was fitted (Kaleidagraph) to the standard single binding site equation which gave a K_d of 2.9 ± 0.5 nM and B_{max} of 0.039 ± 0.001 pmol [^{3}H]-(R)-Rolipram in the presence of 5 mM Mg^{2+}. The data are representative of 3 separate experiments.

placed by Zn^{2+} as the PDE4A activating metal ion (Fig. 4). When the titration of CDP-840 was performed in the presence of both 10 mM Mg^{2+} and 2 μM Zn^{2+}, the IC_{50} was within experimental error of that in the presence of 10 mM Mg^{2+} alone (Fig. 4). These results are consistent with the competition of Mg^{2+} and Zn^{2+} for the activating metal ion site. Since these PDE inhibitors are competitive versus cAMP (14, Z. Huang, unpublished results) and the substrate K_m values in the presence of Mg^{2+} or Zn^{2+} are similar, the increase in IC_{50} values reflect a loss of inhibitor binding affinities upon replacement of Mg^{2+} by Zn^{2+}. Thus the identity of the bound metal ion has a large effect on the affinities of these inhibitors for PDE4A.

PDE4 contains a high affinity ($K_d \approx$1-2 nM), metal ion dependent (R)-Rolipram binding site of low stoichiometry (13). The substantial difference in affinity between this site and the K_i for the inhibition of cAMP hydrolysis (60 nM$<K_i<$1 μM) may be related to the enzyme phosphorylation state (6). In a non-equilibrium filtration binding assay [^{3}H]-(R)-Rolipram bound PDE4A in the presence of Mg^{2+} with a K_d of 2.9 ± 0.5 nM and a B_{max} of 0.005 mol [^{3}H]-(R)-Rolipram/mol enzyme (Fig. 5). In contrast [^{3}H]-(R)-Rolipram binding supported by Zn^{2+} was only slightly greater than in the absence of added metal ion (Fig. 5). At the maximum [^{3}H]-(R)-Rolipram concentration employed (100 nM), binding in the presence of Zn^{2+} was approximately 50% of the maximum observed with Mg^{2+}, indicating a decrease in affinity on the order of 30-fold. It should be noted that in three separate experiments a small, but experimentally significant amount of [^{3}H]-(R)-Rolipram binding ($K_d \approx$ 1-5 nM) in the presence of Zn^{2+} was observed, although the B_{max} was less than 10% that obtained in the presence of Mg^{2+}. Again, Mg^{2+} appears to be competitive with Zn^{2+} since the B_{max} and K_d values for [^{3}H]-(R)-Rolipram binding in the presence of 5 mM Mg^{2+} and 4 μM Zn^{2+} together, were the same as those measured with 5 mM Mg^{2+} alone (data not shown).

DISCUSSION

The identification of the *in vivo* activating metal ion is difficult for enzymes that bind metals loosely ($K_d = 10^{-3}-10^{-8}$ M), resulting in their loss during purification. This is especially so when the isolated enzyme is activated by a variety of metal ions (19). The results of this study clearly show that PDE4A is activated by Zn^{2+}. The Zn^{2+} binding site is likely comprised of one or both of the tandem Zn^{2+}-binding consensus motifs originally identified in thermolysin (7). Homology with this motif has previously identified leukotriene A_4 hydrolase (9), as well as PDE5 (7) and PDE6 (8) as Zn^{2+} hydrolases. A mutagenesis study on PDE4A is also consistent with the importance of these two motifs. Replacement of the two histidines (His433 and His437) of the first motif by serine completely abolished both activity and Rolipram binding, whereas mutation of the second motif (His473 and His477) lead to a large reduction in activity and Rolipram binding (20). Furthermore, mutation of any of the same putative Zn^{2+} ligands in leukotriene A4 hydrolase lead to the expression of protein devoid of both activity and Zn^{2+} (9).

Replacement of Mg^{2+} by Zn^{2+} as activating metal ion lead to large decreases in PDE4A-inhibitor affinities. The plasma level for the therapeutic antidepressant effects of Rolipram is 10-100 nM (21) which is below the IC_{50} value for Rolipram obtained here in the presence of Zn^{2+} and at the low end of the K_d measured in the presence of Zn^{2+}. However, the small sample size of inhibitors tested in this study and the difficulties in correlating purified enzyme IC_{50} values with those from whole cell and animal assays makes it difficult to conclude from these experiments on the likelihood that Zn^{2+} is the PDE4A activating metal ion *in vivo.*

PDE4A is maximally activated by concentrations of Zn^{2+} (0.5-2 μM) which are approximately 3-orders of magnitude lower than the commonly accepted activating metal ion Mg^{2+} (Fig. 1). Intracellular concentrations of Mg^{2+} are approximately 0.3-0.7 mM (22) indicating that PDE4A would not be maximally activated under these conditions. However, phosphorylation of PDE4D by PKA (6) does result in a shift in Mg^{2+} EC_{50} from approximately 3 mM to 0.05 mM, indicating that,

for this isozyme at least phosphorylation may regulate *in vivo* activity. We have not investigated the effect of PDE4A phosphorylation on activation by Zn^{2+} or other metal ions. Interestingly, recombinant PDE4D expressed in E. coli is stimulated by Mn^{2+}, Co^{2+} and Mg^{2+} but is not activated by Zn^{2+} (11). This could reflect the intrinsic properties of the two isoforms, or rather may result from differences in expression system-dependent post translational modifications.

Zinc is present in high concentrations in some tissues although most is bound to proteins such as metallothionein (23). Free intracellular Zn^{2+} concentrations are believed to be in the nanomolar range (24) although micromolar levels have also been reported (25). Thus, intracellular Zn^{2+} concentrations may be sufficient to either support or inhibit PDE4A activity. Previous studies have shown that neutrophil-derived oxidants such as HOCl and NH_2Cl can lead to a mobilization of intracellular Zn^{2+} from metallothionein through the oxidation of coordinating cysteine residues (26, 27). Interestingly, Zn^{2+} has been reported to possess anti-inflammatory activities *in vivo* and *in vitro* (16). It is possible that the increased Zn^{2+} concentrations detected in inflamed tissues may play a role in regulating the inflammatory reaction via the activation or inhibition of PDE4A.

ACKNOWLEDGMENTS

The authors thank M. Ouellet, Z. Huang, D. Pon and D. Riendeau for helpful discussions and D. Pon and C. Brideau for technical assistance.

REFERENCES

1. Beavo, J. A. (1995) *Physiol. Rev.* **75,** 725–748.
2. Conti, M., Nemoz, G., Sette, C., and Vicini, E. (1995) *Endocrine Rev.* **16,** 370–389.
3. Giembycz, M. A., and Kelly, J. J. (1994) *in* Methylxanthines and Phosphodiesterase Inhibitors in the Treatment of Airway Disease (Costello, J. F., and Piper, P. J., Eds.), pp. 27–80, Parthenon, London.
4. Muller, T., Engels, P., and Fozard, J. R. (1996) *TiPS* **17,** 294–298.
5. Hughes, B., Owens, R., Perry, M., Warrellow, G., and Allen, A. (1997) *Drug Discovery Today* **2**(3), 89–101.
6. Sette, C., and Conti, M. (1996) *J. Biol. Chem.* **271,** 16526–16534.
7. Francis, S. H., Colbran, J. L., McAllister-Lucas, L. M., and Corbin, J. (1994) *J. Biol. Chem.* **269,** 22477–22480.
8. Francis, S., Artemeyev, N., Hamm, H., Grimes, K., Sabourin, M., and Corbin, J. *Cell. Biochem. Biophys.*, in press.
9. Medina, J. F., Wetterholm, A., Radmark, O., Shapiro, R., Haeggstrom, J. Z., Vallee, B. L., and Samuelsson, B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7620–7624.
10. Omburo, G. A., Brickus, T., Ghazaleh, F. A., and Colman, R. W. (1995) *Arch. Biochem. Biophys.* **323,** 1–5.
11. Kovala, T., Sanwal, B. D., and Ball, E. H. (1997) *Biochemistry* **36,** 2968–2976.
12. Perry, M. J., O'Connell, J., Walker, C., Crabbe, T., Baldock, D., Russell, A., Lumb, S., Howat, D., Allen, R., Merriman, M., Walls, J., Hughes, B., Higgs, G. A., and Owens, R. J. *Cell. Biochem. Biophys.*, in press.
13. Torphy, T. J., Stadel, J. M., Burman, M., Cieslinski, L. B., McLaughlin, M. M., White, J. R., and Livi, G. P. (1992) *J. Biol. Chem.* **267,** 1798–1804.
14. Wilson, M., Sullivan, M., Brown, N., and Houslay, M. D., (1994) *Biochem J.* **304,** 407–415.
15. Rocque, W. J., Holmes, W. D., Patel, I. R., Dougherty, R. W., Ittoop, O., Overton, L., Hoffman, C. R., Wisely, G. B., Willard, D. H., and Luther, M. A. (1997) *Protein Exp. Purification* **9,** 191–202.
16. Wetterholm, A., Macchia, L., and Haeggstrom, J. Z. (1994) *Arch. Biochem. Biophys.* **311,** 263–271.
17. Kester, W. R., and Matthews, B. W., (1977) *Biochemistry* **16,** 2506–2516.
18. Wang, P., Myers, J. G., Wu, P., Cheewatrakoolpong, B., Egan, R. W., and Billah, M. M. (1997) *Biochem. Biophys. Res. Comm.* **234,** 320–324.
19. Lipscomb, W. N., and Strater, N. (1996) *Chem. Rev.* **96,** 2375–2433.
20. Jacobitz, S., Ryan, M. D., McLaughlin, M. M., Livi, G. P., DeWolf, W. E. Jr., and Torphy, T. J. (1997) *Mol. Pharmacol.* **51,** 999–1006.
21. Torphy, T. J., DeWolf, W. E. Jr., Green, D. W., and Livi, G. P. (1993) *Agents and Actions Supp.* **43,** 51–71.
22. Romani, A., and Scarpa, A. (1992) *Arch. Biochem. Biophys.* **298,** 1–12.
23. Bremner, I. (1991) *Meth. Enzymol.* **205,** 25–35.
24. Denny, M. F., and Atchison, W. D. (1994) *J. Neurochem.* **63,** 383–386.
25. Brand, I. A., and Kleineke, J. (1996) *J. Biol. Chem.* **271,** 1941–1949.
26. Fliss, H., Menard, M., and Desai, M. (1991) *Can. J. Physiol. Pharmacol.* **69,** 1686–1691.
27. Tatsumi, T., and Fliss, H. (1994) *Am. J. Physiol.* **267,** H1597–H1607.