The Human Cyclic AMP-specific Phosphodiesterase PDE-46 (HSPDE4A4B) Expressed in Transfected COS7 Cells Occurs as Both Particulate and Cytosolic Species That Exhibit Distinct Kinetics of Inhibition by the Antidepressant Rolipram*

(Received for publication, March 14, 1996, and in revised form, August 26, 1996)

Elaine Huston, Linda Pooley, Pascale Julien‡, Grant Scotland, Ian McPhee, Michael Sullivan‡, Graeme Bolger¶, and Miles D. Houslay¶

From the Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, IBLS, Wolfson Link Building, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom and VA Medical Center, Huntsman Cancer Institute, Departments of Medicine and Oncologic Science, University of Utah, Salt Lake City, Utah 84148

Transfection of COS7 cells with a plasmid encoding the human cyclic AMP-specific PDE4A phosphodiesterase PDE-46 (HSPDE4A4B) led to the expression of a rolipram-inhibited PDE4 activity, which contributed ~90% of the total COS cell PDE activity. A fusion protein was generated which encompassed residues (758–886) at the extreme C terminus of PDE-46 and was used to generate an antiserum that detected PDE-46 in transfected COS7 cells. Immunoblotting studies identified PDE-46 as a ~125-kDa species that was associated with both the soluble and particulate fractions. The relative V_max of particulate PDE-46 (~56%) that of cytosolic PDE-46. Particulate PDE-46 was not solubilized using Triton X-100 or high NaCl concentrations. Immunofluorescence analysis by laser scanning confocal microscopy showed that PDE-46 was located at discrete margins of the cell, indicative of association with membrane cortical regions. The human PDE4A species, h6.1 (HSPDE4A4C), which lacks the N-terminal extension of PDE-46, was found as an entirely soluble species when expressed in COS7 cells. h6.1 was shown to have an ~11-fold higher V_max relative to that of PDE-46. In dose-response studies rolipram inhibited particulate PDE-46 at much lower concentrations (IC_{50} = 0.195 μM) than those needed to inhibit the cytosolic enzyme (IC_{50} = 1.6 μM). The basis of this difference lay in the fact that rolipram served as a simple competitive inhibitor of the cytosol enzyme (K_i = 1.6 μM) but as a partial competitive inhibitor of the particulate enzyme (K_i = 0.037 μM; K_M = 2.3 μM). Particulate PDE-46 thus showed a ~60-fold higher affinity for rolipram than cytosolic PDE-46.

Cyclic AMP plays a pivotal role in controlling a wide variety

* This work was supported in part by a grant from the Medical Research Council (to M. D. H.) and special equipment and travel grants from the Wellcome Trust and Scottish Office Home and Health Department. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Beatson Institute for Cancer Research, Bearsden, Glasgow G61 1BD, UK.
§ Present address: Astra Charnwood, Bakewell Road, Loughborough, Leicester LE11 ORH, United Kingdom, UK.
¶ To whom reprint requests and all correspondence should be addressed: Division of Biochemistry and Molecular Biology, Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. Tel.: 44-141-330-5903; Fax: 44-141-330-4620; E-mail: gbca29@udcf.gla.ac.uk; WWW: http://www.ibls.gla.ac.uk/IBLSStaff/m-houslay/HouslayLab.html

1 The abbreviations used are: PDE, cyclic AMP phosphodiesterase; PDE4, rolipram-inhibited PDE subfamily; PDE-46, a human PDE4A (HSPDE4A4B); h6.1, a human PDE4A (h-PDE-IVA-h6.1, HSPDE4A4C); h-PDE1, the first human PDE4A to be cloned (HSPDE4A4A); 2EL, inactive human PDE4A splice variant (HSPDE4A4B); RD1, a rat PDE4A (RNPD4A1A); RPDE-6, a rat PDE4A (RNPD4A6A); RPDE-39, a rat PDE4A (RNPD4A8); met(RD1), a form of RD1 lacking the N-terminal first 25 amino acids; rolipram, 4-(3-cyclopentoyl)-4-methoxyphenyl-2-pyrrolidone; PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
the occurrence of alternative mRNA splicing. This, primarily, takes the form of 5'-domain swapping to yield alternatively spliced forms with different N-terminal domains (3), although 2EL, a catalytically inactive human PDE4A variant has been reported (23) that exhibits both a 5’ domain swap together with an insertion toward the 3’ end. This results in a frameshift inducing premature truncation (23).

It has been suggested (27–31) that the function of the alternatively spliced N-terminal domains of the rat PDE4A family may be to allow for membrane/cytoskeletal association through protein-protein interaction and also regulation of enzyme activity through manipulation of the V<sub>max</sub> of the enzyme. Here we analyze the properties of the human PDE4A species PDE-46 (HSPDE4A4B) when expressed in COS-7 cells. We show that this enzyme is expressed in both cytosolic and particulate/membrane compartments and that these two forms differ markedly as regards their catalytic activity (V<sub>max</sub>) and the mechanism of inhibition by the selective PDE4 inhibitor and antidepressant rolipram. We also show that h6.1 (HSPDE4A4AC), which lacks the N-terminal extension seen in PDE-46, is expressed in the cytosol where it is ~11-fold more active than cytosolic PDE-46.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, Dulbecco’s modified Eagle’s medium, and fetal calf serum were from Life Technologies, Inc. (Paisley, UK). Tris, Heps, DEAE-dextran (M<sub>w</sub> 500,000), cytochalasin B, benzamidine hydrochloride, phenylmethylsulfonyl fluoride, aprotenin, peptatin A, antipain, EDTA, EGTA, cyclic AMP, cyclic GMP, Dowex 1-X8-400 (chloride form, 200–400 mesh), 3-isobutyl 1-methylxanthine, snake venom (Ophiophagus hannah), phospho-buffered saline, isopropl-1-thio-β-D-galactopyranoside, ampicillin, glutathione, and bovine brain calmodulin were from Sigma (Poole, UK). [3H]Cyclic AMP and [3H]cyclic GMP were from Amersham International (Amersham, UK). pGEX-3X was from Pharmacia (UK) Ltd. Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, UK). Dithiothreitol, Trion X-100, and lysozyme were from Boehringer (UK) Ltd. (Leusew, UK). Triethanolamine was from BDH (Glasgow, UK). Glycerol was from Fison (Leicestershire, UK). Bradford reagent was from Bio-Rad (Hertfordshire, UK). Dimethyl sulfoxide was from Koch-Light Ltd (Haverhill, UK). Rolipram was a generous gift from Schering Aktiengesellschaft, Postfach 650311, D-1000 Berlin 65, Germany. MTT was from Calbiochem. 

Generation of a Plasmid Allowing the Expression of PDE-46 in COS Cells—The cloning of PDE-46 and generation of a plasmid containing the entire open reading frame of this PDE4A enzyme has been reported previously by one of us (16). The entire open reading frame for PDE-46 was excised from the p-BlueScript plasmid containing the PDE-46 cDNA as a 3.59-kilobase pair fragment using SpeI and NoI. This was ligated into the vector pSV-SPORT and then cut using SmaI and SpeI to generate the plasmid pSV-SPORT-pde46.

COS Cell Expression Plasmid for h6.1—pSV-SPORT-h6.1, the plasmid containing the cDNA for h6.1, and “vector only” pSV-SPORT-APX were generated as described previously by us (32).

Transfection of COS-7 Cells—COS-7 cells were seeded at approximately 1/3 confluency onto 10-cm diameter plates, 18 h before the transfection. Immediately before transfection the culture medium was replaced with 5 ml of Dulbecco’s modified Eagle’s medium (Life Technologies, Inc. Europe, Glasgow) supplemented with 10% (v/v) Nuserum (Collaborative Biomedical Products) together with 0.1 mM chloroquine. Transfection—Immediately before transfection the culture medium was replaced with 5 ml of Dulbecco’s modified Eagle’s medium (HSPDE4A4C), which lackstheN-terminalextensionseenin PDE-46. Peptides were synthesized with a cysteine at the N terminus and were generated as described previously by us (27, 28, 30). This procedure routinely yielded a P1 pellet (1,000 × g, 10 min) and a P2 pellet (60 min at 100,000 × g) as well as a high speed supernatant (S). The homogenization procedure was complete in that there was no detectable latent lactate dehydrogenase activity present in the P1 pellet, indicating an absence of cytosol proteins.

Generation of a Fusion Protein Formed Between Glutathione S-Transferase (GST) and a C-terminal Region of Human PDE4A—A 300-bp DNA fragment encoding amino acids 788–886 of the human PDE4A species PDE46 (GenBank™, accession no. L28469), was generated by using PCR primers 5’-pde46, 5’-Ctgggatccgagctgccacc-3’; and 3’-pde46, 5’-GTCACGATGATCCTCAGGAGGTCCTCC-3’. These were designed so as to contain recognition sites for the restriction enzymes BamHI and EcoRI, respectively. The plasmid pSV-SPORT-6.1 (32) formed the template in the following reaction: 1 μg of template DNA, 25 pmol of each primer, 0.2 mM each dNTP, 50 mM KC1, 10 mM Tris-HCl (pH 9.0), 0.1% Trion X-100, 1.5 mM MgCl<sub>2</sub> in a final volume of 50 μl with a mineral oil overlay. The reaction was subjected to the following conditions in a Techne PHC-3 thermocycler: denaturation for 1' at 94 °C, annealing for 2 min at 37 °C, extension for 3 min at 72 °C for one cycle followed by denaturation for 1' at 94 °C annealing for 2 min at 60 °C, extension for 3 min at 72 °C for 30 cycles. Upon completion, the reaction was digested with an enzyme of choice and the resulting product was cloned into the BamHI/EcoRI sites of the inducible bacterial expression vector pGEX-3X. This created an in-frame fusion with the GST gene in a plasmid which we have called pGEX-3X-(788–886)-PDE46.

Generation of a Polyclonal Antiserum to the GST-(788–886)-PDE4A Fusion Protein—Escherichia coli cultures (JM110), transformed with either pGEX-3 or recombinant pGEX-3X-(788–886)-PDE46, were first grown overnight at 37 °C with agitation in Luria-Bertani medium containing 50 mg/ml ampicillin, diluted 1:10 in the same medium, and incubated at 37 °C. Fusion protein expression was induced at 37 °C by adding 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and growth was continued at 37 °C for 6 h. Bacteria were centrifuged, suspended at a 1:10 dilution in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 100 mM NaF, and 1% Nonidet P-40, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Brilliant Blue staining.

New Zealand White rabbits were immunized by subcutaneous injection with 120 μg of the fusion protein GST-(788–886)-PDE46 in complete Freund’s adjuvant and boosted twice at monthly intervals in incomplete Freund’s adjuvant. These antisera were referred to as PAb42/GST-(788–886)-PDE46. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Brilliant Blue staining.

Confoanal Analyses—48 h after transfection, COS-7 cells were plated out onto coverslips (18 × 18 mm) at about 60% confluency. After another 24 h the cells were fixed to the coverslips using paraformalde-
Expression of PDE4A in COS Cells

Hyde and labeled with antiserum followed by staining with goat anti-(IgG) rhodamine conjugate (TCS Biologicals) as described previously. Control experiments were done using cells which were treated with (i) preimmune antiserum and (ii) goat anti-(IgG) rhodamine B conjugate and no primary antibody, and (iiii) by using cells which had not been detergent-permeabilized with TX-100. Experiments were performed on four separate transfection studies in each instance. The data shown are typical of the cells analyzed. For each transfection, single optical sections were taken for $10$ cells with full galleries of images obtained for five cells where $x$-$y$ optical sections were collected in the $z$ plane through the entire cell at $0.2$-$\mu m$ intervals.

The microtubule network was visualized using a mouse monoclonal antibody raised against $\alpha$-tubulin. This antibody (34) is a generous gift from Prof. Keith Gull, School of Biological Sciences, University of Manchester, UK.

Analyses were done using a Zeiss laser scanning confocal microscope using an Axiovert 100 microscope with a $\times 63/1.4NA$ plan apochromat lens. A $543$-nm laser line was selected with appropriate filters (LP$570$; KT $488/543$) and a pinhole size of $8$–$14$. Three-dimensional reconstruction was done using either Zeiss LSM software on an IBM Pentium system or using Imaris (2.2.4) software (Bitplane AG, Zurich; Fairfield Imaging Ltd., East Sussex, UK).

**RESULTS AND DISCUSSION**

**Human PDE4A Species**—Three cloned, active human PDE4 enzymes have been reported (16, 17, 32). Of these, PDE-46, which was cloned by one of us (16), is the largest species (Fig. 1), has been shown to express cyclic AMP specific PDE activity when been shown to express cyclic AMP specific PDE activity when expressed in a mammalian system we engineered the cDNA encoding the entire open reading frame which was cloned by one of us (16), is the largest species (Fig. 1) and is believed to provide a full-length product of the PDE4A gene, being analogous to the rat PDE4A splice variant RPDE-6 (3, 16, 30, 31). The activity of a truncated form of PDE-46 has been shown to express cyclic AMP specific PDE activity when expressed in Saccharomyces cerevisiae (16). In order to determine the activity and properties of the full-length product of the PDE4A gene when expressed in a mammalian system we engineered the cDNA encoding the entire open reading frame of PDE-46 into the pSV-SPORT vector for expression in COS cells.

h6.1 is a PDE4A species reported on by some of us previously (32). This was generated from two overlapping cDNA clones whose sequence encompassed a region which is nearly identical to that found within PDE-46 (16). This region is from the amino acid residue at position 210 of PDE-46 to the extreme C-terminus of PDE-46 (Fig. 1). It thus encompasses the entire putative catalytic region (16) together with the C-terminal region of PDE-46 but lacks the N-terminal region of PDE-46, which, in the rat PDE4A gene (Fig. 1), has been shown to be alternatively

---

**Figure 1. Schematic representation of PDE4A forms.** This demonstrates the relationship between PDE-46 (HSPEDE4A1A; GenBank™ accession no. L209965), h6.1 (HSPEDE4A4C, GenBank™ accession no. U18087), h-PDE1 (HSPEDE4A4A; GenBank™ accession no. M37744), and 2EL, an inactive human PDE4A splice variant (HSPEDE4A7; GenBank™ accession no. U18088). The cognate positions of the two splice junctions found for the rat PDE4A gene are shown (i and ii), as are the introns conserved regions (16). The rat PDE4A splice variants RD1 (RNPD4EA1A; GenBank™ accession no. M26715), RPDE-6 (RNPD4EA5; GenBank™ accession no. L72057) and RPDE-39 (RNPD4EA8; GenBank™ accession no. L36467) are shown together with the engineered species metRD1, a form of RD1 lacking the N-terminal first 25 amino acids (27, 28). The position of the putative catalytic region is indicated (3, 6).
Expression of PDE4A4 in COS Cells

33137

spliced (3). h-PDE1 was the first human PDE4A species to be reported (17), and this contains the region encompassed by the two clones we used to generate h6.1 (32) together with 9 additional amino acids at its N terminus, which were preceded by an initiator methionine (Fig. 1). To generate a PDE that would be expressed, we employed (32) a PCR-based strategy in order to add an initiator methionine together with the N-terminal 9 additional amino acid residues reported in h-PDE1 (17). This generated the reported clone h6.1 which thus bears close similarity to h-PDE1 (17). However, h-PDE1 (17) differs from h6.1 (32) in having certain base changes, which would lead to five differences in amino acids found in and around the putative catalytic region of the protein and which have been suggested (32, 39) might account for the differences in rolipram inhibition kinetics exhibited by these two forms. Excluding the engineered region, the nucleotide sequence of h6.1 (32) exactly matches that which can be found in the human PDE4A genomic sequence and, with one base difference, is identical to that reported for the cognate region of PDE46 (16). It is thus possible that the bases encoding the N-terminal 9 residues of h-PDE1 (17) and those that imply other differences may have resulted from cloning/sequencing artifacts.

A complex series of splice variants appear to be produced from the rat PDE4A gene (Fig. 1) and these all take the form of N-terminal domain swapping (3, 31). The situation for the human PDE4A gene is less well developed although, in addition to PDE46 (16), a splice variant, called 2EL, has been identified (Fig. 1), which exhibits no apparent PDE activity (23). PDE-46 (16) provides a distinct PDE4A splice variant which is expressed natively in cells. Thus transcripts have been identified in a variety of tissues by RNase protection (16) and in MonoMac 6 cells by Northern blotting (40). Indeed, immunoblotting (40) of MonoMac 6 cells for PDE4A forms identified a single immunoreactive species of ~130 kDa, which bears comparison with that found in COS cells transfected to express PDE-46 (see below). In contrast, to our knowledge, h-PDE1 (17) has not been shown to be expressed natively. Nevertheless, at a minimum, h-PDE1 (17) and h6.1, the species we generated (32, 39) to mimic it, provide useful representations of what we might presume, from analogy with the rat PDE4A splice variants and the species met26RD1 (27) (Fig. 1), as the “core” human PDE4A gene product (16, 27, 30, 31, 41). Indeed, in this regard h6.1 (32), as with the rat PDE4A species, which has the engineered deletion of the N-terminal splice regions, met26RD1 (27, 28, 30), is found as a soluble, cytosolic species when transiently expressed in COS-1 cells.

Expression of PDE-46 Activity in COS-7 Cells—Transfection of COS-7 Cells with the plasmid pSV-SPORT-pde46 increased the cAMP phosphodiesterase activity of the homogenate such that <5% of the PDE activity was attributable to endogenous enzymes. Typically, PDE activities were in the range 8–12 nmol/min/mg of protein using 1 μM cAMP as substrate (n = 5 experiments using different transfections). Mock transfection with the parent plasmid (pSV-SPORT) had no effect (3%) on either the total endogenous COS7 cell PDE activity or the fraction of that which was inhibited by rolipram (10 μM). The increase in PDE activity seen in pSV-SPORT-pde46-transfected cells was unaffected by the addition of either Ca2+/calmodulin (100 μM, 20 ng ml−1; <5% change), which would stimulate any PDE1 activity, or by 1 μM cGMP (<5% change), which would alter either PDE2 or PDE3 activities (1, 5). This increase in homogenate PDE activity was, however, severely attenuated (>92%) by the addition of rolipram (10 μM rolipram at 1 μM cAMP), which serves as a selective inhibitor of PDE4 enzymes.

The increase in PDE activity subsequent to transfection with a plasmid encoding PDE-46 was distributed between both particulate and cytosol compartments, with the majority of the activity being in the cytosolic fraction. Thus we noted that some 88 ± 3% (errors are S.D.; n = 4) of the increase in PDE activity was associated with cytosol and the rest with the particulate/membrane fraction. Activities in both of these fractions exhibited similar low Km values for cAMP hydrolysis (Table I). These are similar to the values reported for other PDE4 enzymes (3, 6, 7, 13, 14, 16–19, 31, 32, 39, 42, 43).

Immunological Detection of PDE-46—We have previously been able to generate specific antisera to rat PDE4A and PDE4B species using dodecapeptides representing the extreme C-terminal sequence of these enzymes (28, 33). We were, however, singularly unsuccessful here in trying to generate such antiseria useful in either Western blotting or immunoprecipitation. This was done employing both a peptide whose sequence reflected that found at the C-terminal end of both PDE-46 and h6.1 and one which represented an internal sequence of PDE-46 (data not shown; see “Experimental Procedures”). Thus, in order to try and obviate this problem we generated a GST-fusion protein so as to be able to use a larger fragment of PDE-46/h6.1 as an immunogen. This involved using PCR to generate a DNA fragment encoding amino acids at the extreme C terminus (788–886) of PDE-46 and fusing it, in frame, to GST. The production of this fusion protein was determined by SDS-PAGE (Fig. 2). Using this species as an immunogen we raised antiseria which were able to detect both GST and the pde46-GST fusion protein (Fig. 2). The antibodies directed at GST itself could be removed by treatment of the antiserum with immobilized GST, leaving a treated antiserum which now recognized only the fusion protein (Fig. 2).

This showed that we had been able to generate antibodies which recognized epitopes within the PDE-46/h6.1 region of the fusion protein. Such a treated antiserum was used in subsequent experiments, although similar results were obtained in Western blotting studies using the untreated antiserum to which excess GST was added before use in immunoblotting studies (data not shown). Indeed, as a routine we added excess GST to all immunoblotting studies even when the purified antiserum was employed.

Antiseria, raised against the C-terminal human PDE4A-GST fusion protein, allowed us to detect (Fig. 2) a single immuno-

---

**Table I**

| Assay* | Cytosolic PDE-46 | Particulate PDE-46 | h6.1* |
|--------|------------------|-------------------|-------|
| K<sub>m</sub> (μM) | 2.6 ± 0.6 (4) | 1.9 ± 0.3 (4) | 6 ± 2 (6)** |
| K<sub>i</sub> (μM) | 1.6 ± 0.3 (4) | 0.037 ± 0.008 (4) | 0.4 ± 0.1 (3)* |
| K<sub>i</sub> (μM) | N/A | 2.3 ± 0.9 (4) | N/A |
| Relative V<sub>max</sub> (1) | 0.54 ± 0.08 (7) | 11.5 ± 3.5 (9) | 1.0 ± 0.2 (3)* |
| IC<sub>50</sub> (μM) | 1.6 ± 0.3 (4) | 0.195 ± 0.035 (4) | 0.6 ± 0.2 (3)* |

* K<sub>m</sub> and V<sub>max</sub> were derived from computer fitting to the Michaelis Menten equation (see “Experimental Procedures”). K<sub>i</sub> was derived graphically by linear regression analysis (see legend to Fig. 7) and K<sub>i</sub> was by nonlinear iterative least squares fitting to Equation 2 (see “Experimental Procedures”).

h6.1 data are taken either from Sullivan et al. (32) alone* or additional data (three observations)** was pooled together with that published previously for h6.1. * N/A, not applicable.

---

* M. Sullivan, A. Olsen, and M. D. Houslay, manuscript in preparation.
Expression of PDE4A in COS Cells

PREVIOUSLY, WE HAVE EXPRESSED H6.1 IN COS1 CELLS (32) AND SHOWN THE INCREASED ROLIPRAM-INHIBITED PDE4 ENZYME ACTIVITY TO BE FOUND IN THE CYTOSOL FRACTION BUT NOT ASSOCIATED WITH THE HIGH-SPEED (P2) MEMBRANE FRACTION. SIMILARLY, HERE WE OBSERVED THAT TRANSFECTION OF COS7 CELLS WITH pSV.SPORT-H6.1 LED TO INCREASED ROLIPRAM-INHIBITED PDE4 ACTIVITY LOCALIZED IN THE HIGH-SPEED SUPERNATANT CYTOSOL FRACTION WITH NO OBSERVABLE INCREASE IN THE PDE ACTIVITY OF THE MEMBRANE FRACTION (<5% CHANGE; N = 4). CYTOSOLIC EXPRESSED H6.1 PDE EXHIBITED A SPECIFIC ACTIVITY OF 1.5 ± 0.4 nmol CAMP HYDROLYZED/MIN/ML OF PROTEIN (N = 4). ALL OF THIS INCREASED PDE ACTIVITY (96%) COULD BE INHIBITED BY ROLIPRAM (10 μM ROLIPRAM WITH 1 μM cAMP AS SUBSTRATE). CONSISTENT WITH SUCH ACTIVITY STUDIES, IMMUNOBLOT ANALYSIS ALSO SHOWED THAT, UPON TRANSFECTION WITH pSV.SPORT-H6.1, THEN A 99 ± 3 KDA (S.D.; N = 4) IMMUNOREACTIVE SPECIES WAS EVIDENT IN THE CYTOSOL FRACTION BUT NOT IN THE MEMBRANE PELLET (FIG. 1). AS WITH PDE46, h6.1 EXPRESSED IN COS7 CELLS EXHIBITED AN APPARENT MOLECULAR SIZE, UPON SDS-PAGE, WHICH WAS GREATER THAN THE VALUE OF 76.4 KDA DEDUCED FROM ITS PRIMARY SEQUENCE (32).

That h6.1 was found as a soluble, cytosolic species when expressed in COS cells, whereas PDE46 was found in both particulate-associated and soluble forms, indicates that the N-terminal extension of PDE46 contains information that allows anchoring to particulate/membrane fractions. This situation bears analogy to the protein products of the rat PDE4A

reactive species in both the cytosol and particulate fractions of COS cells which had been transfected with the vector pSV.SPORT-pde46 but not in those cells which had been transfected with a control vector (pSV.SPORT). The immunoreactive species detected in both locations exhibited identical sizes upon SDS-PAGE with molecular masses of 125 ± 4 kDa and 124 ± 4 kDa for the cytosol and particulate forms, respectively (N = 4, separate experiments; S.D.). When these species were run together on the same gel, they co-migrated with each other and with the single species found using a homogenate extract from pSV.SPORT-pde46-transfected COS cells (data not shown). Preincubation of these antisera with the C-terminal PDE4A-GST fusion protein prevented the detection of such an immunoreactive species (data not shown). The size of the immunoreactive species, seen in pSV-SPORT-pde46-transfected cells, was greater than that predicted (99.2 kDa) for PDE-46 on the basis of its primary sequence (16). However, our studies done on the three established rat PDE4A splice variants RD1 (30), RPDE-6 (30), and RPDE-39 (31) showed that all these enzymes exhibited slower migration (i.e. larger apparent size) on SDS-PAGE than might be predicted simply from their sequence. This may be due to folding or to stretches of acidic amino acids reducing the amount of SDS bound to the protein. Furthermore, the sequence of the human PDE-46 shows considerable similarity to that of the rat PDE4A splice variant RPDE-6, a species which when expressed in COS cells migrates on SDS-PAGE with an apparent molecular size of −109 kDa compared to a calculated size of −94 kDa (3, 30). As with the increase in PDE4 activity observed in pSV.SPORT-pde46-transfected COS cells, an immunoreactive species was evident in both membrane and cytosol fractions (Fig. 2).

Both PDE-46 activity and immunoreactivity from membranes of pSV.SPORT-pde46-transfected COS cell was not released (<5%) upon treatment with either NaCl concentrations up to 1.5 M or with concentrations of the detergent Triton X-100 up to 5% (data not shown). This bears analogy with the rat PDE-6 and rat PDE-39 PDE4A splice variants (30, 31), but not with the rat RD1 splice variant which, although being exclusively membrane-associated, was readily solubilized by the detergent Triton X-100 (27, 30). Such treatments of these particulate fractions did not affect PDE activity or its susceptibility to inhibition by rolipram (data not shown).

The availability of antisera able to detect PDE-46 allowed us to determine the relative amounts of the enzyme in the isolated particulate and cytosol fractions of transfected COS7 cells. From this we were able to determine the relative Vmax values for these two populations of PDE-46 (Table I). Such analyses showed that the Vmax of the particulate form of PDE-46 was 56 ± 9% of that of the cytosolic form (errors are S.D.; n = 4 separate experiments).

Expression of h6.1 in COS7 Cells—Previously we have expressed h6.1 in COS1 cells (32) and shown the increases rolipram-inhibited PDE4 activity to be found in the cytosol fraction but not associated with the high-speed (P2) membrane fraction. Similarly, here we observed that transfection of COS7 cells with pSV.SPORT-h6.1 led to increased rolipram-inhibited PDE4 activity located in the high-speed supernatant cytosol fraction with no observable increase in the PDE activity of the membrane fraction (<5% change; n = 4). Cytosolic expressed h6.1 PDE exhibited a specific activity of 1.5 ± 0.4 nmol CAMP hydrolyzed/min/ml of protein (n = 4). All of this increased PDE activity (96%) could be inhibited by rolipram (10 μM rolipram with 1 μM cAMP as substrate). Consistent with such activity studies, immunoblot analysis also showed that, upon transfection with pSV.SPORT-h6.1, then a 99 ± 3 kDa (S.D.; n = 4) immunoreactive species was evident in the cytosol fraction but not in the membrane pellet (Fig. 1). As with PDE46, h6.1 expressed in COS7 cells exhibited an apparent molecular size, upon SDS-PAGE, which was greater than the value of 76.4 kDa deduced from its primary sequence (32).
gene. The PDE4A gene thus appears to encode a “core,” highly active, soluble protein to which various N-terminal extensions can be spliced. These N-terminal regions allow interaction with membrane/particulate fractions, generating species which are either totally membrane-associated or distributed between cytosol and membrane compartments (27, 28, 30, 31). In this regard, in transfected COS-7 cells 25% of the cognate species to PDE-46, namely RPDE-6, was found to be associated with the membrane/particulate fraction (30). Thus the N-terminal regions of both the human and rat PDE4A enzymes appear to play a targeting role.

The availability of antisera able to detect both PDE-46 and h6.1 has allowed us to determine their relative concentrations in extracts from transfected COS cells. This was done using 125I-labeled second antisera (Fig. 2B). From this we were able to determine the relative V max values for h6.1 compared to cytosolic PDE-46, which expresses a large N-terminal splice domain (Table I). This showed that h6.1 had a considerably higher (~11.5-fold) maximal activity compared to that of PDE-46.

Laser Scanning Confocal Microscopy Analyses of Transfected COS Cells—COS cells were transfected so as to express either h6.1 or PDE-46, fixed, permeabilized, and challenged with specific antiserum and rhodamine-labeled anti-rabbit antiserum before analysis of their immunofluorescence using laser scanning confocal microscopy as described under “Experimental Procedures.” No fluorescent signal was evident in cells which had either not been permeabilized, indicating that the PDE signal was indeed intracellular, or where no primary antibody had been added or if an excess of blocking GST-PDE4A fusion protein had been added (see “Experimental Procedures”; data not shown). On the basis of immunoblotting and activity studies, levels of any endogenous PDE4A would be extremely low (<1%) compared to the levels of PDE-46 and h6.1 seen in transfected COS cells. Consistent with this, transfected cells were clearly distinguishable as highly fluorescent species set among the presumed nontransfected cells, which showed an extremely low and poorly resolved signal (data not shown).

In COS cells transfected so as to express h6.1 we observed a clear fluorescent signal which extended throughout the cytosol of these cells. This was evident from optical sections viewed in the x-y plane cutting across the middle of the COS cells (Fig. 3). Obtaining multiple images in the z plane allowed for the reconstruction of cellular fluorescence in three dimensions and, from this, z-x and z-y slices down through the cell could be generated (Fig. 3). These showed that the fluorescent signal due to h6.1 permeated through the entire cytosol of the transfected cells but was clearly excluded from the nucleus (Fig. 3). However, the intensity of immunofluorescence due to h6.1 appeared to be somewhat asymmetrically distributed through the cytosol, with increased intensity levels seen in the cytosol region that was closest to the nucleus (Fig. 3). In this regard, such a region around the nucleus was shown to be highly enriched in bundles of microtubules (Fig. 3). Interestingly, the pattern of fluorescence observed with h6.1 differed from that seen (28) for the engineered (27) core-soluble rat PDE4A form, metSRD1 which was distributed evenly throughout the cytosol of transfected COS cells. This might indicate that h6.1 can associate with localized structures inside the cell but in a reversible fashion, which is disrupted upon cell breakage to release soluble h6.1.

A strikingly different pattern of immunofluorescence was obtained using COS cells that had been transfected so as to express PDE-46. In x-y sections taken horizontally through the middle of the cells then concentrated areas of fluorescence were found at the cell periphery (Fig. 4). This was supported from both z-x and z-y slices down through the cell which, again, highlighted a concentration of fluorescence at the cell margin as well as an evident fluorescent signal permeating through the cell cytosol (Fig. 4). That the highest immunofluorescence was observed at the cell margin indicates that the relative concentration of PDE-46 at such a location must be considerably higher than the concentration of PDE-46 found within the cytosol. However, from biochemical analyses done on disrupted cells, by far the greatest amount of PDE-46 was found as a soluble species. One explanation may be that particulate-associated PDE-46 achieves a much higher local concentration than that of the pool of soluble PDE-46, which is distributed throughout the cytosol. The fluorescence at the cell periphery was, however, not evenly distributed. Rather, it appeared to occur at distinct margins of the cell that are suggestive of association with cortical structures, including lamellae and pseudopods (44, 45). In this regard, such immunofluorescence patterns are similar to those described for fodrin (nonerythroid spectrin) and the actin binding protein, cortactin (44, 45). In
Expression of PDE4A in COS Cells

indeed, if PDE-46 was to interact with cytoskeletal or cytoskeletal-associated proteins this might explain why particulate PDE-46 was not solubilized by detergent or high salt treatment. This distribution of fluorescence due to PDE-46 was very different from that seen from the rat PDE4A RD1 when expressed in COS cells (28). RD1 has a very different N-terminal region to PDE-46 and is found as an exclusively membrane-associated species (30) in transfected COS cells, where it appears to be associated with both the Golgi apparatus, and the cytosol surface of the plasma membrane, where a distinct punctate pattern of fluorescence was observed (28). This suggests that alternative splicing may provide N-terminal regions that allow for distinct targeting of PDE4 enzymes within the cell.

Inhibition of Particulate and Cytosol Forms of PDE-46 by Rolipram—Rolipram caused the dose-dependent inhibition of both particulate and soluble PDE-46 forms expressed in pSVSPORT-pde46-transfected COS-7 cells (Fig. 5). However, distinct differences in these dose-effect curves were evident in that the particulate activity appeared to be more sensitive to inhibition by rolipram, exhibiting an IC50 value of 0.195 ± 0.035 μM rolipram, compared with the cytosolic form which exhibited an IC50 value of 1.6 ± 0.3 μM rolipram (n = 4 separate experiments; errors as S.D.). In view of this we undertook a detailed study of the inhibition of the particulate and cytosolic forms of PDE-46 by rolipram. From such studies we were able to determine that rolipram served as a simple competitive inhibitor of the cytosolic enzyme (Fig. 6). Thus, double reciprocal activity plots, performed at different rolipram concentrations, showed a common intersection on the y axis (Fig. 6) and produced linear slope replots against rolipram concentration (Fig. 6) and linear Dixon plots (data not shown) (46). From these we were able to derive Ki values of ~1.6 μM for the association of rolipram with cytosolic PDE-46 (Table I). Such a value is slightly greater than that recorded for h6.1 where, similarly, rolipram acts as a simple competitive inhibitor (32, 39).

Double reciprocal plots of particulate PDE activity, done at different rolipram concentrations, exhibited a common intersection on the y axis (Fig. 7A). However, in marked contrast to the action of rolipram on the soluble enzyme, in this instance, for the particulate enzyme, we noted that both the slope replots (Fig. 7A) and the Dixon plots (46) were nonlinear (Fig. 7B). The parabolic nature of these two plots is consistent with rolipram serving as a partial competitive inhibitor of the particulate enzyme (47) (see Scheme 1).

\[
\begin{align*}
E & \rightleftharpoons ES & \rightarrow & E + \text{products} \\
K_1 & \uparrow I & K_2 & \uparrow I & k_6 \\
E1 & \rightleftharpoons EIS & \rightarrow & E1 + \text{products} \\
\end{align*}
\]

\text{SCHEME 1}
Expression of PDE4A in COS Cells

Such graphical analyses of the kinetic data for the particulate enzyme (Fig. 7C) show quite clearly that these predictions hold true with both such plots being markedly nonlinear. A further prediction of such a kinetic mechanism is that double reciprocal plots of the change in slope (Δ slope of the double reciprocal plot data) against inhibitor (rolipram) concentration should be linear, as implied by Equation 4.

$$\frac{1}{\Delta \text{slope}} = \frac{1}{V_{\max}} \cdot \frac{K_i \cdot (1 + [\text{rolipram}]/K_i)}{[\text{cAMP}] \cdot (1 + ([\text{rolipram}]/K_i))}$$

(Eq. 4)

Graphical analysis shows that this, indeed, appears to be the case for the particulate enzyme (Fig. 7C). Indeed, from such a plot the affinity constant ($K_i$) for rolipram binding to the enzyme-substrate complex (ES) can be determined from the intercept on the x axis (Fig. 7; Table I). Using these data it is then possible to employ curve-fitting routines applied to the slope replot data (Fig. 7) in order to determine values for the association of rolipram to the free particulate enzyme ($K_i$) and also the constant for substrate (cAMP) association with enzyme ($K_s$) (Table I). Thus the inhibitor affinity constant for the free particulate enzyme ($K_i$) was seen to be (Table I) considerably lower than that for the substrate-bound particulate enzyme ($K'_i$). Furthermore, the $K_i$ value for the particulate form of PDE-46 was considerably lower than that observed for the cytosolic form (Table I), indicating that rolipram bound more tightly to particulate PDE-46 than it did to the cytosolic form of this enzyme. This explains why, in dose-effect studies, that the particulate enzyme began to be inhibited at much lower concentrations of rolipram than those which exerted actions on the cytosolic enzyme (Fig. 5).

For cytosolic PDE-46, which obeys simple competitive kinetics, then saturating concentrations of rolipram (≥10 μM) will completely (>99%) inhibit PDE activity, and such an effect was observed experimentally (>98% inhibition). However, for particulate PDE-46, which obeys kinetics of partial inhibition, then the inhibitor-bound enzyme is able to hydrolyze cAMP, and thus rolipram will be unable to obliterate the entire particulate PDE-46 activity. From the experimentally determined kinetic constants (Table I), it is possible to calculate, using Equation 1, the magnitude of the residual activity PDE expected in the presence of 10 μM rolipram while using 1 μM cAMP as substrate. This activity would be expected to form ~6% of that observed in the absence of added rolipram. Such a value is consistent with experimental data showing that 10 μM rolipram can inhibit PDE activity assayed with 1 μM cAMP by ~92% (Fig. 5). Such an altered kinetic mechanism may result as a consequence of PDE-46 becoming associated with membrane/particulate fractions. Indeed, there is a precedent for this where the solubilization of monoamine oxidase led to an alteration in kinetic mechanism (48).

Skying is intriguing, however, that the particulate form of PDE-46 showed a markedly higher affinity for rolipram (~62-fold) than

$$\frac{1}{V_{\max}} \cdot \frac{K_s \cdot (1 + [\text{rolipram}]/K_s)}{[\text{cAMP}] \cdot (1 + ([\text{rolipram}]/K_s))}$$

(Eq. 3)

where $v_{\text{obs}}$ is the initial rate, $V_{\max}$ is the maximum velocity of the reaction, $K_s$ is the affinity of the enzyme for substrate (cAMP), $K_i$ is the affinity constant which reflects binding of inhibitor (rolipram) to the free enzyme, and $K'_i$ is the affinity constant which reflects binding of inhibitor (rolipram) to the enzyme-substrate complex.

As can be seen from Equation 2, a prediction of this form of mechanism (47) is that the slope replots from double reciprocal plots of the initial rates of PDE activity against [cAMP] done at different rolipram concentrations will be nonlinear.

$$\text{Slope} = \frac{K_s \cdot (1 + [\text{rolipram}]/K_s)}{V_{\max} \cdot (1 + ([\text{rolipram}]/K_s))}$$

(Eq. 2)

Similarly, as can be deduced from Equation 3, the Dixon (46) replots of the reciprocal of the initial rates of PDE activity against rolipram concentration will be nonlinear.

In this situation inhibitor can bind not only to free enzyme ($E$), as with a simple competitive inhibitor, but it can also bind to the enzyme-substrate complex ($ES$). For apparent competitive inhibition to be realized, however, the $ES$ complex has to hydrolyze cAMP at a rate which is identical (experimentally indistinguishable) from that at which the $ES$ complex hydrolyzes cAMP, i.e., the rate constants $k_s = k_e$. This is because a partial competitive inhibitor does not change the $V_{\max}$ of the reaction (47). Partial competitive inhibitors thus yield double reciprocal plots of initial rates of substrate utilization against substrate concentration done at different inhibitor concentrations, which are identical to those seen for full competitive inhibitors: that is, they are linear and show no effect on $V_{\max}$ but an increase in apparent $K_m$. This is self-evident from the rate equation (Equation 1) for partial competitive inhibition which is given by

$$v_{\text{obs}} = \frac{V_{\max}}{1 + \left(\frac{K_s}{[\text{cAMP}] + [\text{rolipram}]K_i}\right)}$$

(Eq. 1)

where $v_{\text{obs}}$ is the initial rate, $V_{\max}$ is the maximum velocity of the reaction, $K_s$ is the affinity of the enzyme for substrate (cAMP), $K_i$ is the affinity constant which reflects binding of inhibitor (rolipram) to the free enzyme and $K'_i$ is the affinity constant which reflects binding of inhibitor (rolipram) to the enzyme-substrate complex.

FIG. 6. Inhibition of the soluble form of PDE-46 by rolipram. Double reciprocal plots are shown for PDE activity against cAMP substrate concentration given at increasing rolipram concentrations with the slope replot given as an inset. The rolipram concentrations featured in the double reciprocal plot were none [●], 0.1 μM [○], 0.5 μM [□], 1 μM [▲], 3 μM [■], 5 μM [◇], and 10 μM [●]. PDE activity is expressed in nanomoles/min/mg of protein. These are typical sets of data from experiments done four times using different transfections.

where

$$\frac{1}{V_{\max}} \cdot \frac{K_i \cdot (1 + [\text{rolipram}]/K_i)}{[\text{cAMP}] \cdot (1 + ([\text{rolipram}]/K_i))}$$

(Eq. 3)
the soluble enzyme, as indicated from the differences in their $K_i$ values (Table I). In this regard, various investigators have reported on the existence of high affinity binding sites for rolipram with association constants between one and two or- 
orders of magnitude lower than the IC$_{50}$ values reported for the rolipram inhibition of soluble PDE4 preparations (49–52). One possible contributor to this is likely to be PDE4D3, which shows a markedly enhanced susceptibility to inhibition by rolipram when it is phosphorylated on a unique site in its N- 
terminal splice region by protein kinase A (20). However, it is possible that particulate-associated PDE-46 may provide a contribution to the population of high affinity rolipram binding sites by virtue of the alteration in the kinetics of rolipram inhibition of this PDE4A splice variant. It is, however, important to appreciate that because of the partial competitive nature of rolipram inhibition of particulate PDE4A, the magnitude of the affinity of this fraction of the enzyme for rolipram will be severely underestimated in studies done trying to make inferences about this by determining IC$_{50}$ values at a single concentration of cAMP. This is because such a value will reflect

**Fig. 7. Inhibition of the particulate form of PDE-46 by rolipram.** In A is shown a double reciprocal plot for PDE activity against cAMP substrate concentration given at increasing rolipram concentrations the slope replot data given as an inset. The parabolic line drawn through this data was generated by a fitting routine (see "Experimental Procedures") set to use Equation 2. The rolipram concentrations featured in the double reciprocal plot were none (□), 0.1 μM (●), 0.5 μM (○), 1 μM (▲), 3 μM (■), 5 μM (△), and 10 μM (▲). In B is shown the Dixon replot of the data from A with the reciprocal of the reaction velocity against [rolipram] given at increasing cAMP concentrations. The parabolic lines drawn through each set of data were generated by a fitting routine (see "Experimental Procedures") set to use Equation 3. The cAMP concentrations featured in this plot were 1 μM (●), 1.5 μM (□), 2 μM (●), 3.5 μM (△), 5 μM (■), and 10 μM (▲). In C is shown double reciprocal plots of the change in slope (delta slope) from the inset of A against the inhibitor (rolipram) concentration. A linear regression line could be fitted to these data with $r^2 > 0.995$, and this was used to determine the value of $K'_i$ as per Equation 4 (47). PDE activity is expressed in nanomoles/min/mg of protein. These are typical sets of data from experiments done four times using different transfections.
Expression of PDE4A in COS Cells

...not only the high affinity binding of rolipram to the free enzyme but also the lower affinity binding of rolipram to the cAMP-bound enzyme.

In studies done using h-PDE1, expressed in _S. cerevisiae_ and in baculovirus-infected insect cells, anomalous rolipram binding kinetics were observed together with high affinity rolipram binding (53, 54). Intriguingly, however, using h6.1 expressed in _S. cerevisiae_, rigorous kinetic analyses served only to show that simple competitive kinetics of inhibition by rolipram ensued (39). It has been suggested (39) that the differences in inhibition between these two forms might reflect differences in the sequence of these forms at residues in and around the catalytic region. Indeed, it has been shown (55) that single amino acid changes in a PDE4 enzyme can lead to profound changes in sensitivity to rolipram inhibition. As the sequence of h6.1, other than the 9 residues at its N terminus, reflects that of PDE4, it is possible that residue changes seen in h-PDE1 might have elicited a conformation change which mimics that adopted by the particulate form of PDE-46. It is, however, possible that, in contrast to h6.1, when h-PDE1 was expressed in _S. cerevisiae_ it became modified in a manner which triggered a similar change in conformation to that exhibited by the particulate form of PDE-46, resulting in altered kinetics of rolipram inhibition.

The molecular cloning of the _dunc_ PDE from _Drosophila_ has served as a paradigm for the PDE4 PDE family. However, an interesting anomaly is that this enzyme was apparently not inhibited by rolipram (14, 16, 31, 56, 57). This may be due to structural differences in the binding site and, indeed, in this regard it has been demonstrated (55) that single amino acid changes in PDE4 can dramatically affect the ability of rolipram to serve as an inhibitor. However, it is possible that rolipram might in fact bind to the _Drosophila dunc_ enzyme without inhibiting it if the _dunc_ enzyme obeyed kinetics of partial competitive inhibition. For, under conditions where the affinity of both free enzyme and the enzyme-substrate complex for rolipram was identical (_K_′ = _K_′) then, as was deduced by Dixon (47) from Equation 1, no apparent inhibition of enzyme activity would result.

CONCLUSIONS

Transfection of COS7 cells with a plasmid encoding the PDE4A splice variant PDE4E demonstrates, as seen for the rat homologue RPDE-6 (30), both cytosolic and particulate forms. Here we show that particulate PDE-46 is highly localized to cortical areas of the cell. Intriguingly, the two populations of PDE-46 demonstrate very different susceptibilities to inhibition by rolipram. This appears to reflect changes in their kinetic mechanisms of inhibition and the conformation of the active site. In this regard the maximum activity of the particulate form is only about half that of the cytosol form. We suggest that PDE-46 can exist in two conformationally distinct states which here are reflected in the cytosolic and particulate populations. The PDE4 selective inhibitor rolipram thus appears to serve as an effective detector of these two states, which can be characterized by changes in both the kinetics of inhibition and the affinity of interaction with rolipram. While the functional significance of these two states and whether PDE4E can dynamically switch between them remains to be determined, their existence has implications for the design and application of inhibitors for therapeutic use. The biological significance of the targeting of PDE-46 remains to be elucidated. It may relate to the anchoring of protein kinase II isozymes (58, 59) so as to effect the compartmentalization of cAMP signaling within the cell, or it could be so as to confer specific regulatory properties upon the immobilized PDE fraction. Thus modification of the N-terminal region of PDE-46 and alterations in its interactions with anchoring/binding proteins may well have profound regulatory consequences.

REFERENCES

1. Beavo, J. A., Conti, M., and Haslippi, R. J. (1994) Mol. Pharmacol. 46, 399–405
2. Beavo, J. A. (1995) Physiol. Rev. 75, 725–748
3. Bolger, G. (1994) Cell. Signalling 4, 851–859
4. Houslay, M. D., and Kilgour, E. (1990) (Beavo, J. A., and Houslay, M. D., eds) Second Messenger Phosphoprotein Res. 25, 87–99
5. Thompson, W. J. (1991) _Pharmacol. Ther._ 51, 13–33
6. Houslay, M. D., Griffiths, S. L., Horton, Y. M., Livingstone, C., Lobban, M., Macdonald, P. Morris, N. Pye, J., Scotland, G., Shukur, Y., Sweeney, G., and Tang, E. K. Y. (1992) _Biochem. Soc. Trans._ 20, 140–146
7. Reeves, M. L., Leigh, B. K., and England, P. J. (1987) _Biochem. J._ 241, 535–541
8. Thompson, W. J., Epstein, P. M., and Tipton, K. F. (1974) _J. Biol. Chem._ 249, 645–652
Expression of PDE4A in COS Cells

Marshall, S. C., Marfat, A., Masamune, H., Shirley, J. T., Tickner, J. E., and Umland, J. P. (1995) *Bioorg. Med. Chem. Lett.* 5, 1969–1972

50. Masamune, H., Cheng, J. B., Cooper, K., Eggler, F., Marfat, A., Marsh, S. C., Shirley, J. T., Tickner, J. E., Umland, J. P., and Vazquez, E. (1995) *Bioorg. Med. Chem. Lett.* 5, 1965–1968

51. Schneider, H. H., Schmiechen, R., Brezinski, M., and Seidler, J. (1986) *Eur. J. Pharmacol.* 127, 105–115

52. Tohda, M., Murayama, T., Hasegawa, H., Nogiri, S., and Nomura, Y. (1994) *Neurosci. Lett.* 175, 89–91

53. 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

54. Amegadzie, B. Z., Hanning, C. R., McLaughlin, M. M., Burman, M., Cieslinski, L. B., Livi, G. P., and Torphy, T. J. (1995) *Cell Biol. Int.* 19, 477–484

55. Pillai, R., Staub, S. F., and Colicelli, J. (1994) *J. Biol. Chem.* 269, 30676–30681

56. Davis, R. L. (1988) *Methods Enzymol.* 159, 786–792

57. Cherry, J. A., and Davis, R. L. (1995) *J. Neurobiol.* 28, 102–113

58. Scott, J. D., and McCartney, S. (1994) *Mol. Endocrinol.* 8, 5–11

59. Rubin, C. S. (1994) *Biochim. Biophys. Acta* 1224, 467–479