Identification of two splice variant forms of type-IV_B cyclic AMP phosphodiesterase, DPD (rPDE-IV_{B1}) and PDE-4 (rPDE-IV_{B2}) in brain: selective localization in membrane and cytosolic compartments and differential expression in various brain regions

Margaret LOBBAN,* Yasmin SHAKUR,* James BEATTIE† and Miles D. HOUSLAY*‡

*Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and †Hannah Research Institute, Ayr, Scotland, U.K.

In order to detect the two splice variant forms of type-IV_B cyclic AMP phosphodiesterase (PDE) activity, DPD (type-IV_{B1}) and PDE-4 (type-IV_{B2}), anti-peptide antisera were generated. One set ('DPD/PDE-4-common'), generated against a peptide sequence found at the common C-terminus of these two PDEs, detected both PDEs. A second set was PDE-4 specific, being directed against a peptide sequence found within the unique N-terminal region of PDE-4. In brain, DPD was found exclusively in the cytosol and PDE-4 exclusively associated with membranes. Both brain DPD and PDE-4 activities, isolated by immunoprecipitation, were cyclic AMP-specific ($K_m^{cyclicAMP}$: ~ 5 μM for DPD; ~ 4 μM for PDE-4) and were inhibited by low rolipram concentrations ($K_1^{rolipram}$ ~ 1 μM for both). Transient expression of DPD in COS-1 cells allowed identification of an approx. 64 kDa species which co-migrated on SDS/PAGE with the immunoreactive species identified in both brain cytosol and membrane fractions using the DPD/PDE-4-common antisera. The subunit size observed for PDE-4 (approx. 64 kDa) in brain membranes was similar to that predicted from the cDNA sequence, but that observed for DPD was approx. 4 kDa greater. Type-IV, rolipram-inhibited PDE activity was found in all brain regions except the pituitary, where it formed between 30 and 70% of the PDE activity in membrane and cytosolic fractions when assayed with 1 μM cyclic AMP. PDE-4 formed 40–50% of the membrane type-IV activity in all brain regions save the mid-brain (approx. 20%). DPD distribution was highly restricted to certain regions, providing approx. 35% of the type-IV cytosolic activity in hippocampus and 13–21% in cortex, hypothalamus and striatum with no presence in brain stem, cerebellum, mid-brain and pituitary. The combined type-IVB PDE activities of DPD and PDE-4 contributed approx. 10% of the total PDE activity in most brain regions except for the pituitary (zero) and the mid-brain (approx. 3%). The isolated cDNAs for DPD and PDE-4 appear to reflect transcription products which are expressed *in vivo* in brain. The unique N-terminal domain of PDE-4 is suggested to target this PDE to membranes in brain. Type-IVB PDEs are differentially expressed in various brain regions, indicating that there are tissue-specific controls on both the expression of the gene and the splicing of its products.

INTRODUCTION

Cyclic AMP serves as a second messenger which plays a pivotal role in controlling cell functioning. The sole means of its inactivation is through the action of cyclic AMP phosphodiesterases (PDE) which hydrolyse cyclic AMP to 5'AMP (Beavo, 1990; Manganiello et al., 1990; Houslay and Kilgour, 1990; Conti and Swinnen, 1990; Conti et al., 1991). This is a large enzyme family encoded by multiple genes with the added complexity of multiple splicing (Beavo, 1990; Conti and Swinnen, 1990; Davis, 1990). The type-IV PDE family specifically hydrolyse cyclic AMP in a fashion which, unlike type-II and type-III PDE isoforms, is insensitive to low concentrations of cyclic GMP and which, unlike type-I PDE activity, is insensitive to Ca^{2+}/calmodulin (Beavo, 1990; Conti and Swinnen, 1990; Davis, 1990; Conti et al., 1991). The first type-IV isoforms to be purified to apparent homogeneity were from dog kidney (Thompson et al., 1979) and rat liver (Marchmont et al., 1981). However, full appreciation of the diverse nature of the type-IV PDE isoforms arose from studies done on learning-defective mutants of *Drosophila* where it was found that the *dunc* gene encoded a type-IV PDE (Chen et al., 1986; Davis, 1990). Cognate mammalian cDNAs were isolated using the *dunc* gene as a probe of both rat (Davis et al., 1989; Swinnen et al., 1989; Conti and Swinnen, 1990; Davis, 1990; Conti et al., 1991) and human (Livi et al., 1990; Mchale et al., 1991; McLaughlin et al., 1993; Bolger et al., 1993) cDNA libraries and also using complementation in an engineered yeast strain (Colicelli et al., 1989).

The various rat and human type-IV enzymes that have now been characterized, subsequent to molecular cloning, share another property in addition to their specificity for hydrolysing cyclic AMP in a cyclic GMP-insensitive fashion. This is an ability to be specifically and selectively inhibited by rolipram (see e.g. Conti et al., 1991), a compound which causes sedation of behaviour in rats (Wachtel, 1983a,b), consistent with the anti-depressant actions that it exhibits in man (Wachtel, 1983a; Guit-Goffioul et al., 1987; Hebenstreit et al., 1989). This suggests that at least certain of these type-IV PDEs may be responsible for

Abbreviations used: PDE, cyclic AMP phosphodiesterase; RD1, rat '*dunc*-like' PDE (also referred to as rPDE-IV_{A1} and with a new Gene Bank locus proposed as RPDE4A1B represents rat type-IV PDE isoform subfamily A splice variant 1); DPD, '*dunc*-like PDE' (also referred to as rPDE-IV_{B1} and has a new Gene Bank locus proposed as RPDE4B1); PDE-4, splice variant of the PDE type-IV_B family which is also referred to as rPDE-IV_{B2} and has a new Gene Bank locus proposed as RPDE4B2A; IBMX, isobutylmethylxanthine; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidone; Met^{26}-RD1, RD1 with the N-terminal 25 residues deleted.

‡ To whom correspondence should be addressed.

mediating the effects of rolipram on central nervous system function. Certainly transcripts for various PDE-IV isoforms have been identified in rat brain by Northern-blot analyses (Davis et al., 1989; Swinnen et al., 1989). This implies the expression of such isoforms in brain and is consistent with observations that rolipram can both inhibit a soluble brain PDE extract having the characteristics of type-IV activity (Nemoz et al., 1985) and can also bind specifically to protein sites in rat brain membrane and cytosolic fractions (Schneider et al., 1986).

It is now generally accepted that there are four type-IV PDE isoform families, called IV_A, IV_B, IV_C and IV_D (Beavo, 1990). From rat, full-length cDNAs have been isolated for IV_{A1} (RD1), IV_{B1} (DPD) and IV_{B2} (PDE-4) (see Davis, 1990; Conti and Swinnen, 1990; Conti et al., 1991) although it appears that further multiplicity may occur through alternative splicing (Monaco et al., 1994; Bolger, 1994) and ambiguity in the use of start codons. To our knowledge, however, nothing is known about whether the proteins predicted from the various cDNAs reflect those of identifiable type-IV PDE proteins which are expressed *in vivo* and whether they are differentially expressed within different brain areas. This work attempts to address such issues by using specific anti-peptide sera generated to amino acid sequences predicted from the cDNAs of rat type-IV_B PDEs: namely DPD (PDE-IV_{B1}) (Colicelli et al., 1989) and PDE-4 (PDE-IV_{B2}) (Swinnen et al., 1989). (In this work the type-IV isoforms are referred to in terms of the 'colloquial' names given to them by those who first described these cDNAs. In parentheses, however, we have also given details of the unifying descriptors suggested by Beavo (1990) and the latest proposals for revision in the descriptors used to describe cDNA clones reported to GenBank.) These two cDNAs are believed to reflect splice variants of the products of a single gene (Monaco et al., 1994) where absolute identity is predicted for the protein products except for an N-terminal extension of 48 residues seen in PDE-4, the role of which is unknown.

MATERIALS AND METHODS

Materials

Tris, Hepes, DEAE-dextran (500 kDa), cytochalasin B, benzamidine hydrochloride, phenylmethanesulphonyl fluoride (PMSF), aprotinin, pepstatin A, antipain, EDTA, EGTA, cyclic AMP, cyclic GMP, Dowex 1X8-400 (chloride form, 200–400 mesh), 3-isobutyl-1-methylxanthine, snake venom (*Ophiophagus hannah*), and bovine brain calmodulin were from Sigma Chemical Co. (Poole, Dorset, U.K.). [^{3}H]Cyclic AMP and [^{3}H]cyclic GMP were from Amersham International (Amersham, Bucks., U.K.). Leupeptin was from Peptide Research Foundation (distributed by Scientific Marketing Associates, London, U.K.). Dithiothreitol, Triton X-100, and Triton X-114 were from Boehringer (U.K.) (Lewes, U.K.). Triethanolamine was from BDH (Glasgow, U.K.). Glycerol was from Fisons (Loughborough, Leics., U.K.). Bradford reagent was from Bio-Rad (Herts., U.K.). Dimethyl sulphoxide (DMSO) was from Koch-Light Ltd. (Haverhill, U.K.). Rolipram was a generous gift from Schering Aktiengesellschaft (Berlin, Germany). Marker proteins (prestained protein molecular-mass markers 14.3–200 kDa range were from BRL). The cDNA encoding DPD was a generous gift from Dr. M. Wigler (Cold Spring Harbor Laboratory, Cold Spring Harbor, Long Island, U.S.A.).

Preparation of rat brain membranes and soluble fractions

Intact brains were removed from six 200–250 g male Sprague–Dawley rats immediately after death, rapidly dissected into eight sub-regions, namely: pituitary, brain stem, cerebellum, hippocampus, striatum, cortex, mid-brain and hypothalamus. Each of these was, immediately upon isolation, placed into ice-cold homogenization buffer and, within a few minutes, homogenized using eight strokes at full speed on a rotary homogenizer using a Teflon pestle and glass vessel. This was done on ice using a 1:10 (w/v) dilution of sectioned brain and homogenization buffer. The homogenization buffer contained 20 mM Tris/HCl (final pH 7.2), 1 mM EDTA and 0.25 M sucrose together with a protease inhibitor cocktail of 0.1 mM PMSF, 2 mM benzamidine, 2 mM aprotinin, 2 mM pepstatin A, 2 mM leupeptin and 2 mM antipain. Each homogenate was centrifuged at 1000 $g_{av.}$ for 10 min. The supernatant was decanted and centrifuged at 100000 $g_{av.}$ for 1 h. This yielded a soluble fraction and a membrane pellet. The latter was resuspended in ice-cold homogenization buffer to the same volume as the soluble fraction. These fractions were either analysed immediately or 'snap-frozen' in 0.5–1.0 ml aliquots, using liquid N_2, and stored at −80 °C. Similar results were obtained in both instances. Frozen aliquots were not subjected to refreezing and frozen samples were not stored beyond 7 days.

Fractionation studies were also done in some instances. In such experiments, homogenization of whole brain was performed as described above. However, the homogenate so formed was then centrifuged at 1000 $g_{av.}$ for 10 min to yield a P1 pellet. The supernatant was decanted, before being recentrifuged at 100000 $g_{av.}$ for 1 h to yield a P2 pellet together with a supernatant (S) fraction. The membrane pellets were each individually resuspended in ice-cold homogenization buffer and then analysed.

Preparation of antisera

Two peptides were used to raise polyclonal anti-PDE sera in rabbits. One of these was chosen to reflect a sequence (Colicelli et al., 1989; Swinnen et al., 1989) found at the extreme C-terminus of both DPD and PDE-4, namely (C)-A^{508}-T-E-D-K-S-L-I-D-T^{517}, on the basis that this might be expected to generate a 'DPD/PDE-4-common' antiserum. In addition, we identified a sequence, (C)-G^{6}-T-V-S-G-A-G-S-S-R-G-G-G^{18}, which is unique to PDE-4 and is found at the extreme N-terminus (Colicelli et al., 1989; Swinnen et al., 1989). This was selected on the basis that it might allow for the generation of PDE-4-specific antisera. In selecting such sequences we were careful to insure that in neither instance were they found in any of the other forms of rat PDE-IV which have so far been identified (Conti and Swinnen, 1990). The peptides were synthesized with a cysteine (C) at the C-terminus in order to facilitate conjugation to keyhole limpet haemocyanin (KLH), as described in Lerner (1981). Antiserum was normally added to KLH–Sepharose and mixed overnight at 4 °C before being centrifuged briefly to pellet the Sepharose. The supernatant was collected and dialysed overnight against 20 mM Tris/HCl (final pH 8)/1.4 M NaCl at 4 °C. The antisera used in this study are referred to as 'DPD/PDE-4-common' and 'PDE-4-specific'. In each instance the sera from two rabbits were used with up to five different bleeds with essentially identical results being obtained.

SDS/PAGE

Acrylamide gels (8 % or 10 %) were used and the samples boiled for 3 min. after being resuspended in Laemmli (1970) buffer. Gels were run at 8 mA/gel overnight or 50 mA/gel for 4–5 h with cooling.

Western-blotting procedure

As a routine, samples (100 μg of protein) of the various rat brain region membrane and cytosol fractions were separated by SDS/PAGE as above. These were transferred to nitrocellulose for immunoblotting using individual antisera as described by us previously for various protein kinase C and G-protein antisera (Bushfield et al., 1990; Palmer et al., 1992; Tang et al., 1993). Labelled bands were identified using anti-(rabbit peroxidase-linked IgG) and the Amersham enhanced chemiluminescence (ECL) Western blotting visualization protocol or ^{125}I-labelled anti-(rabbit IgG). Quantification of the relative amounts of antigen present in each of the fractions was done in a variety of ways, as described before (Bushfield et al., 1990; Palmer et al., 1992; Tang et al., 1993), in order to identify situations where a linear relationship held between the amount of antigen present and the magnitude of the signal detected. As a routine, densitometric scanning of the ECL experiments was done using both photosensitive Amersham film and X-ray film with various exposure times and a range of applied protein concentrations so as to identify a linear detection range. Confirmation was also obtained in selected instances using ^{125}I-labelled anti-(rabbit IgG) with excision of the labelled bands identified by autoradiography, for counting, as described previously by us (Bushfield et al., 1990; Palmer et al., 1992; Tange et al., 1993). Peptide competition experiments were done as described by us previously (Tang et al., 1993).

Certain PDEs are very sensitive to proteolysis (Beavo, 1990; Houslay and Kilgour, 1990). However, using the isolation and homogenization conditions detailed herein, we believe that little, if any, proteolysis of these PDEs occurred, in that reproducible patterns of expression and relative amounts of PDE activities were evident in the various brain areas investigated and no immunoreactive species of lower molecular mass were identified.

Membrane solubilization

Membranes (100 μg protein; 1 vol.) were taken up in 10 vol. of ice-cold solubilization buffer. This contained 1% (v/v) Triton X-100 together with 10 mM EDTA, 100 mM NaH_2PO_4, 50 mM Hepes buffer, final pH 7.2, and the protease inhibitor 'cocktail' described earlier. Samples were briefly vortexed and then left on ice for 1 h, with occasional mixing, in order to effect solubilization. After this, the samples were centrifuged for 1 h at 100000 $g_{av.}$ at 4 °C. The supernatant fraction was used as a source of solubilized PDE activity. Such a procedure routinely solubilized > 96% of the PDE activity in the membranes.

Immunoprecipitation of PDE activity

Solubilized membrane fractions (100 μg of starting material; see above) and cytosol (supernatant) fractions, which had been diluted 1:10 (v/v) in solubilization buffer containing protease inhibitors (see above), were used as sources of material for the specific immunoprecipitation of PDE activity. Antiserum was added to each sample at a dilution of 1:100 and the samples briefly vortexed before being left overnight (~ 16 h) at 4 °C in order to allow conjugation to proceed; no change in PDE activity ensued over such a period, nor did any change occur in the immunoreactive species identified by Western blotting. Pansorbin (100 μl per 800 μl of sample) was then added and the resultant suspension gently mixed at 4 °C for 2 h. The preparations were then centrifuged at 14000 $g_{av.}$ for 2 min with the pellets being resuspended in the solubilization buffer before being recentrifuged at 14000 $g_{av.}$ for 2 min. The resultant pellets were then washed again twice. The final pellet was resuspended in PDE assay buffer (20 mM Tris/HCl, 10 mM $MgCl_2$, final pH 7.4) and assayed immediately.

In brain extracts from the various regions we also noted that increasing the amount of antisera added in the immunoprecipitation experiments, from a 1:100 to a 1:50 dilution, had no effect (< 5%) on the amount of PDE activity that was immunoprecipitated, nor did it affect the PDE activity.

Generation of an expression vector for DPD

DPD cDNA was excised from pADPD (a generous gift from Dr. M. Wigler, Cold Spring Harbor, U.S.A.) as an *Eco*RI/*Hin*dIII fragment. It was then ligated to *Eco*RI/*Hin*dIII-cut pBluescript KS$^-$ to give the plasmid pKS-DPD. DPD cDNA was then released as a *Xho*/*Bam*H1 fragment and ligated to *Xho*/*Bam*H1-cut pSVL. This generated the expression vector pSVL-DPD for the transient transfection of COS cells.

Expression of DPD in COS-1 cells

The culture, transfection and homogenization of COS-1 cells were performed as described in some detail by us previously (Shakur et al., 1993).

PDE assay

PDE activity, using 1 μM cyclic AMP as substrate, was assayed by a modification of the two-step procedure of Thompson and Appleman (1971) and Rutten et al. (1973) as described previously by Marchmont and Houslay (1980). All assays were conducted at 30 °C and in all experiments a freshly prepared slurry of Dowex/water/ethanol (1:1:1, by vol.) was used for determination of activities. In all the experiments described initial rates were taken from linear time courses of activity. Rolipram was dissolved in 100% DMSO as a 10 mM stock solution and diluted in 20 mM Tris/HCl, 10 mM $MgCl_2$ buffer (final pH 7.4) to provide a range of concentrations for use in the assay. The residual levels of DMSO were shown not to affect PDE activity over the ranges used in this study.

Protein determination

Protein was routinely measured by the method of Bradford (1976) using BSA as a standard.

RESULTS AND DISCUSSION

Localization to membrane and cytosol fractions

Blotting cortex homogenates with anti-(DPD/PDE-4-common) sera identified an immunoreactive band (Figure 1a) occurring at 64 $\pm$ 2 kDa. This was evident (Figure 1a) in the low-speed (P1) and high-speed (P2) pellets as well as the supernatant fraction (S), suggesting that both membrane-associated and soluble cytosolic type-IV$_B$ PDEs were present in brain. Recognition of the immunoreactive band was blocked by the inclusion of the peptide used to generate the antiserum but not by an unrelated species, indicating a specific interaction (Figure 1c). Such antisera did not identify any immunoreactive species in mock (or pSVL 'vector only')-transfected COS-1 cells (results not shown). However, in COS-1 cells transfected with pSVL-DPD they identified a species which co-migrated with that identified in brain (track DPD; Figure 1a). These antisera can thus identify DPD and indicate that it migrates with an apparent molecular mass which is somewhat higher than that which would be predicted (61 kDa) from the sequence of the cDNA (Colicelli et al., 1989). This may

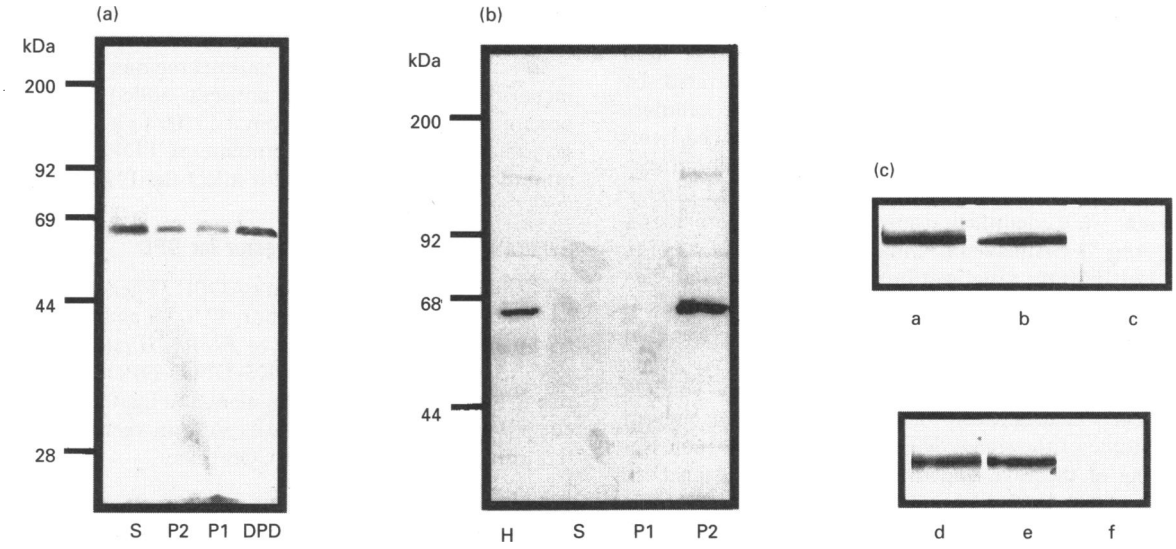

Figure 1 Identification of immunoreactive DPD and PDE-4 in Western blots of cytosol and membrane fractions from cortex

(**a**) Western blot, using DPD/PDE-4-common antiserum of samples (100 μg of protein) from the cytosol (S), low-speed pellet (P1) and high-speed pellet (P2) fractions of brain cortex. Also shown is a cytosol extract of COS-1 cells which had been transfected with DPD-pSVL so as to express DPD. In COS cells so transfected the supernatant PDE activity rose from 15 ± 1 to 193 ± 5 pmol of cyclic AMP produced/mg of protein per min. (**b**) Western blot using PDE-4-specific antiserum of homogenate (H), cytosol (S), low-speed pellet (P1) and high-speed pellet (P2) fractions of brain cortex. (**c**) Western blots of the $\sim$ 64 kDa immunoreactive species identified in cortex cytosol (tracks a, b, c) and solubilized membrane (tracks d, e, f) fractions. Track a, using the DPD/PDE-4-common antiserum alone; track b, in the presence of the PDE-4 N-terminal peptide; and 'track c', with the DPD/PDE-4 C-terminal peptide. Track d, using the PDE-4-specific antiserum alone; track e, with the DPD/PDE-4 C-terminal peptide; and track f, with the PDE-4 peptide. These data show typical experiments of those performed at least three times using different brain preparations and using two sets of antisera.

Table 1 Distribution of DPD and PDE-4 in brain regions

Relative distribution (percentage), in various brain regions, was determined for these two PDE isoforms, namely DPD in the soluble, cytosolic fractions and PDE-4 in the membrane fractions. This was done by (i) subjecting 100 μg of protein from each of the fractions to SDS/PAGE, immunoblotting with designated antisera and then quantifying the amount of immunoreactivity (arbitrary units) as described in the Materials and methods section. These results were summed to give a total with the amount in each fraction then expressed as a percentage of this total in order to gauge a relative specific distribution in each of the fractions. A similar strategy was adopted to determine the relative PDE activity using 1 μM cyclic AMP as substrate. Data obtained using both the PDE-4-specific antiserum and the PDE-4/DPD-common antiserum, for membrane PDE-4, are shown for comparison. Errors are shown as S.D. on the mean for determinations done in three separate experiments using different samples ($n = 3$), with determinations done in triplicate in each instance. Determinations shown as zero indicate failure to observe significant activity or immunoreactivity above background in all three separate experiments.

Preparation	Brain stem	Cerebellum	Cortex	Hippocampus	Hypothalamus	Mid-brain	Pituitary	Striatum
Cytosol								
DPD blot	0	0	36 ± 4	26 ± 3	10 ± 3	0	0	29
DPD activity	2 ± 2	0	33 ± 5	18 ± 4	11 ± 3	0	0	36 ± 5
Membrane								
PDE-4 blot*	4 ± 3	10 ± 3	24 ± 3	20 ± 4	17 ± 4	6 ± 2	0	26 ± 3
PDE-4 activity*	7 ± 2	13 ± 3	20 ± 1	21 ± 4	10 ± 4	7 ± 2	0	22 ± 3
PDE-4 blot**	3 ± 2	9 ± 3	25 ± 3	17 ± 3	15 ± 2	6 ± 2	0	25 ± 5
PDE-4 activity**	12 ± 3	10 ± 3	21 ± 3	23 ± 4	11 ± 2	5 ± 3	0	18 ± 3

*PDE-4 in the membrane fraction gauged using DPD/PDE-4-common antiserum; **PDE-5 in the membrane fraction gauged using PDE-4-specific antiserum.

be due to folding or post-translational modification as, for example, is seen with isoforms of protein kinase C (Marais and Parker, 1989; Pears et al., 1992; Tang et al., 1993). Recent studies on the nature of the PDE-IV_B gene suggest that it is also possible that forms of IV_{B1}, which are larger than the cDNA for DPD, may be produced depending upon the initiator methionine (Monaco et al., 1994) and this may also account for the fact that we observe a species in brain which is larger than that predicted from the cDNA from DPD.

The anti-('DPD/PDE-4-common') antiserum used here was raised against a peptide which reflected a C-terminal sequence found in both DPD and PDE-4; as such, we would predict that it should be capable of interacting with both of these enzymes as PDE-4 only differs from DPD through expressing a unique 48-residue N-terminal extension (Swinnen et al., 1989; Conti and Swinnen, 1990). In order to detect PDE-4 specifically we used, for antisera generation, a conjugated peptide whose sequence lay within the unique N-terminal domain of PDE-4. Using this we identified a single immunoreactive species, migrating at 64 ± 3 kDa, which occurred exclusively in the membrane fraction (Figure 1b). Furthermore, analyses of cytosol and membrane fractions from brain, and also DPD-pSVL-transfected COS cells

Table 2 The relative magnitude of the DPD and PDE-4 activities in various brain regions

Cytosol and membrane fractions were isolated from the various brain areas and each assayed for 'total' PDE activity using 1 μM cyclic AMP as substrate. An approximation of the total type-IV PDE activity was made by determining the degree of inhibition that could be achieved in each fraction using 10 μM rolipram. This acts as a selective inhibitor of type-IV activity at such concentrations (see e.g. Conti and Swinnen, 1990; Conti et al., 1991; Shahid and Nicholson, 1990). In dose–effect studies done on the various fractions, except for pituitary where no inhibition ($< 4\%$) was evident at concentrations up to 100 μM rolipram, inhibition of PDE activity due to rolipram attained a plateau at 10 μM rolipram with half-maximal effects being seen over a range from 1 to 4 μM. The degree of inhibition caused by 10 μM rolipram under such conditions allowed us to express such a 'rolipram-inhibitable component' as a percentage of the total PDE activity; this is referred to as the percentage type-IV PDE activity (% t-IV) in the table. Using antisera to immunoprecipitate DPD and PDE-4 selectively, we then expressed their activities as a percentage of both the type-IV (DPD % t-IV; PDE-4% t-IV) and the total PDE (DPD % Ttl; PDE-4% Ttl) activities in either the cytosol or membrane fractions. Also expressed is the percentage of total PDE activity found in the membrane compared with the cytosolic components; the fraction of the total homogenate activity that the PDE-IV$_B$ family (PDE-4 + DPD) contribute and the fraction of activity that PDE-4 contributes to the PDE-IV$_B$ family (PDE-4 $\pm$ DPD) activity found in various brain regions. Such data are given as means with errors as S.D. for $n = 3$ separate experiments using different brain extracts. Abbreviations: ne, not examined; n/a, not applicable.

	Stem	Cerebellum	Cortex	Hippocampus	Hypothalamus	Mid-brain	Pituitary	Striatum
Cytosol								
% t-IV	35±3	32±8	38±9	30±1	41±3	38±10	ne	36±3
DPD % t-IV	ne	ne	21±3	35±5	17±4	ne	ne	13±3
DPD % Ttl	0	0	8±2	11±2	7±2	0	n/a	5±1
Membrane								
% t-IV	45±9	56±6	55±8	64±7	45±5	44±7	ne	55±6
PDE-4 % t-IV	49±7	46±12	41±8	47±8	40±7	20±3	ne	46±8
PDE-4 % Ttl	22±3	26±7	23±6	30±5	18±3	9±2	n/a	25±4
Homogenate								
Membrane PDE %	50±5	28±5	30±5	16±4	41±7	34±7	38±5	27±3
IV$_B$ % Ttl	11±2	7±1	13±3	14±2	11±2	3±1	0	11±2
PDE-4 % IV$_B$	100	100	55	36	64	100	n/a	64

Table 3 $K_m^{cyclicAMP}$ and $IC_{50}^{rolipram}$ values for immunoprecipitated DPD and PDE-4 from brain cortex

The specific inhibition of DPD and PDE-4 activities was studied using immunoprecipitated enzyme fractions. These used the DPD/PDE-4-common antiserum to isolate DPD from cytosolic fractions of cortex and both the common antiserum and the PDE-4-specific antiserum to isolate PDE-4 from solubilized cortex membranes. $K_m^{cyclicAMP}$ values were determined from Lineweaver–Burk plots (Lineweaver and Burk, 1934) extending over a substrate range from 0.1 to 50 μM cyclic AMP with determinations made at 12 different concentrations of cyclic AMP. Such plots were linear in all instances (correlation coefficient > 0.998 for both regression-line analysis of data and also for non-linear fitting of data to the Michaelis equation; $n = 3$ separate experiments using different preparations). The values for $K_m^{cyclicAMP}$ are given as means with errors as S.D. ($n = 3$). Dose-dependent inhibition by rolipram was determined in the presence of $K_m^{cyclicAMP}$ concentrations of cyclic AMP over a range of rolipram concentrations from 10 nM to 100 μM using 8–10 different concentrations. The concentration of rolipram at which 50% inhibition occurred was determined from such plots and a value for the $K_I^{rolipram}$ calculated assuming competitive inhibition. The values for $K_I^{rolipram}$ are given as means with errors as S.D. ($n = 3$) for separate experiments using different preparations. For comparison, data are also given, from studies done by others, for cDNA species of both DPD and PDE-4 which have been expressed either in yeast or *Escherichia coli*: *Henkel-Tigges and Davis (1990), **Colicelli et al. (1989), ***Swinnen et al. (1989).

PDE analysed	DPD		PDE-4		
Source: antiserum for immunoprecipitation	Brain: anti-DPD/PDE-4	cDNA expressed in yeast	Brain: anti-DPD/PDE-4	Brain: anti-PDE-4	cDNA expressed in *E. coli*
$K_m^{cyclicAMP}$ (μM)	4.8±1.6 ($n = 3$)	2.9*/3.5**	4.4±2.0 $n = 3$)	3.0±1.4 ($n = 3$)	2.5***
$K_I^{rolipram}$ (μM)	0.7±0.2 ($n = 3$)	0.5*	0.55±0.18 ($n = 3$)	0.8±0.3 ($n = 3$)	0.4***

(Figure 1a), with the 'DPD/PDE-4-common' antisera showed a major species which co-migrated in all instances. The mass of the species detected in brain is consistent with that predicted (64.3 kDa; Swinnen et al., 1989) from the sequence of the isolated cDNA for PDE-4, suggesting that this clone does reflect a species which is expressed *in vivo* in brain. Immunoreactivity, using the anti-PDE-4 serum, was blocked if the appropriate competing peptide was included (Figure 1c), indicating that the reaction was specific.

That both sets of antisera identified a single, major immunoreactive band (Figure 1) suggests that further splice variants of the PDE-IV$_B$ class, incorporating the sequences used here to generate anti-peptide sera, are unlikely to occur. We cannot, however, exclude the possibility of variants occurring where such sequences are deleted or where additional residues alter folding to such an extent that the epitopes formed by these peptides are sterically masked.

While our results show that DPD resides in the cytosol, there is a possibility that a fraction might also be membrane-associated. To address this we first investigated COS-1 cells which had been transfected with pSVL-DPD. Upon transfection we noted an increase of some 10–15-fold (range; $n = 3$) in cytosolic PDE activity with no change in the membrane fraction ($< 5\%$); this is consistent with an exclusively cytosolic location for DPD. We were also able to show that this antiserum could be used to immunoprecipitate DPD specifically in a functional state. Thus treatment of the cytosol extract from pSVL-DPD-transfected cells with optimal concentrations of this antiserum allowed for the immunoprecipitation of PDE activity so as to reduce the activity in the supernatant to the level seen in untransfected cells ($\pm 10\%$). No PDE activity was immunoprecipitated if antiserum was added together with the 'DPD peptide' or if cytosol from untransfected cells was used. Thus the specificity for immunoprecipitation paralleled that found for immunoblotting (Figure 1b). Indeed, the PDE activity recovered in the immunoprecipitate was completely inhibited ($> 96\%$) by 10 μM rolipram (1 μM cyclic AMP as substrate) whereas soluble, cytosolic PDE activity in untransfected COS cells was only inhibited some 46±3%

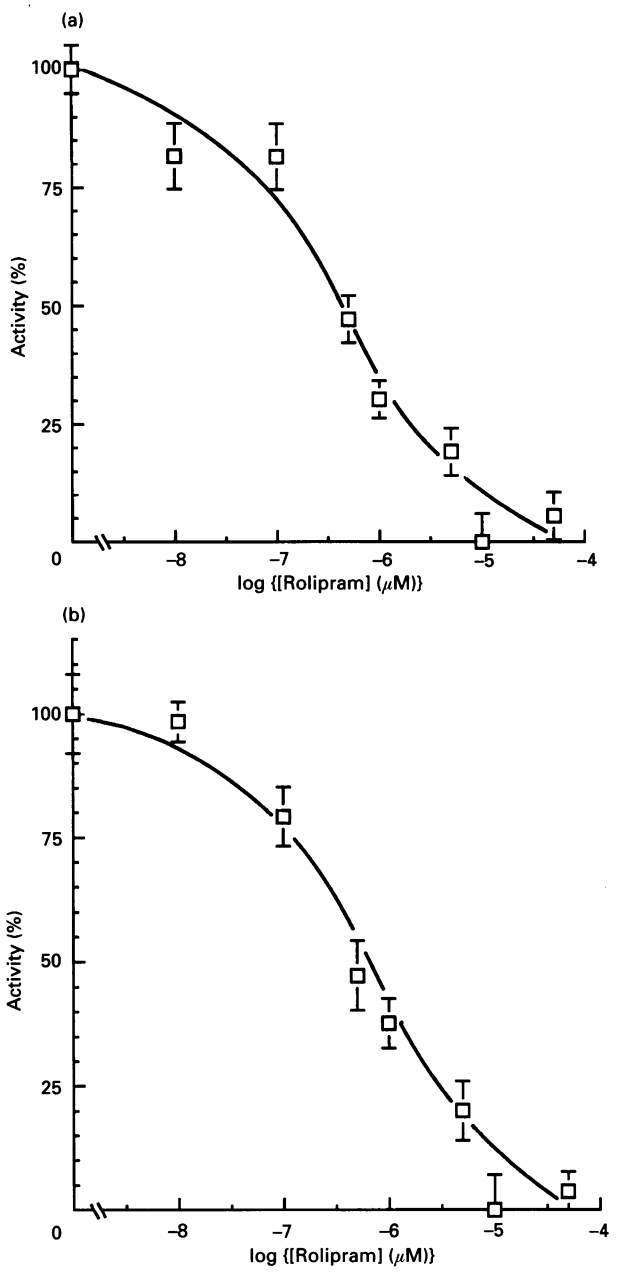

Figure 2 Dose-dependent inhibition of immunoprecipitated DPD and PDE-4 activity by rolipram

As described in the Materials and methods section, brain cortex homogenates were resolved into cytosolic and membrane fractions. DPD was immunoprecipitated from the cytosol and PDE-4 from the solubilized membrane fraction using the DPD/PDE-4-common and PDE-4-specific antisera respectively. The data presented show the dose-dependent inhibition of (**a**) DPD and (**b**) PDE-4 PDE activity in these immunoprecipitates assayed in the presence of 1 μM cyclic AMP together with increasing concentrations of rolipram. Results show means of an experiment done using three separate preparations with means recorded ($n = 3$; $\pm$S.D. indicated by error bars).

($n = 3$) by rolipram. Employing such an immunoprecipitation strategy on soluble cytosolic fractions from brain we found that the DPD/PDE-4-common antisera, but not the anti-PDE-4 sera, caused immunoprecipitation of PDE activity (Tables 1 and 2). In contrast, both antisera were able to immunoprecipitate PDE activity from a detergent-solubilized membrane extract (Tables 1 and 2) which again suggested that PDE-4 was exclusively membrane-bound. In order to test whether a fraction of DPD was membrane-associated in brain, a soluble membrane extract was treated with optimal concentrations of PDE-4-specific antiserum (see the Materials and methods section), whereupon some 20 ± 3% of the PDE activity was immunoprecipitated. However, the subsequent addition of the DPD/PDE-4-common antiserum allowed no further immunoprecipitation of PDE activity to occur, indicating, as in pSVL-DPD-transfected COS cells, the absence of DPD associated with a membrane fraction. We also noted that if the solubilized membrane extract was first treated with the anti-(DPD/PDE-4-common) serum, then a similar fraction of PDE activity was immunoprecipitated (23 ± 4%) to that achieved using the PDE-4-specific antiserum, with subsequent treatment using the PDE-4-specific antiserum unable to immunoprecipitate further PDE activity. These data indicate that DPD resides exclusively in the cytosol and PDE-4 exclusively as a membrane-bound species with targeting presumably occurring due to its unique N-terminal domain, as is also seen for a splice variant of the IV_A family, RD1 (Shakur et al., 1993).

Treatment of cytosolic or solubilized membrane extracts with these antisera failed to alter PDE activity (< 5% difference with either antiserum over the dilutions used for immunoprecipitation), implying that their interaction did not elicit any conformational change in the catalytic site. This may be because the epitopes are located in small regions at the extremities of these PDE species which are well away from the putative catalytic domain (Charbonneau, 1990; Jin et al., 1992).

The activities immunoprecipitated by these antisera were typical of type-IV PDEs in that they did not hydrolyse cyclic GMP (< 1% rate with cyclic AMP) and the inclusion of either a low concentration of cyclic GMP (1 μM) or Ca^{2+}/calmodulin (100 μM; 20 ng/ml) in the assays failed (< 4% change) to affect activity (1 μM cyclic AMP as substrate). As reported for the expressed cDNAs in model systems (Table 3), these activities were also completely inhibited by the specific inhibitor rolipram (Figure 2; Table 3) in a dose-dependent fashion with submicromolar values for $K_i^{rolipram}$ and exhibited $K_m^{cyclicAMP}$ values in the low micromolar range (Table 3).

Localization within specific brain regions

Immunoblot analysis of soluble cytosolic and total membrane fractions, from various brain regions, with both PDE-4-specific antisera and DPD/PDE-4-common antisera, indicated that both DPD and PDE-4 were selectively expressed in different brain regions (Figure 3).

The various brain regions could be divided into a number of distinct groups based upon their expression of DPD and PDE-4 immunoreactivity (Figure 3; Table 1). Most striking was the pituitary where no immunoreactive material was evident in either the cytosol or the membrane fractions, indicating the absence of both DPD and PDE-4. A group comprising the cerebellum, brain stem and mid-brain was characterized by the absence of cytosolic DPD and the presence of membrane-bound PDE-4; and a group formed from the striatum, hypothalamus, hippocampus and cortex had both membrane-bound PDE-4 and cytosolic DPD expressed. An identical distribution, for membrane immunoreactivity, was found using both the PDE-4-specific antisera and the DPD/PDE-4-common antiserum (Figure 2; Table 1).

Using specific immunoprecipitation we determined the distribution, in various brain regions, of the activities of cytosolic DPD and membrane-bound PDE-4. These closely followed those

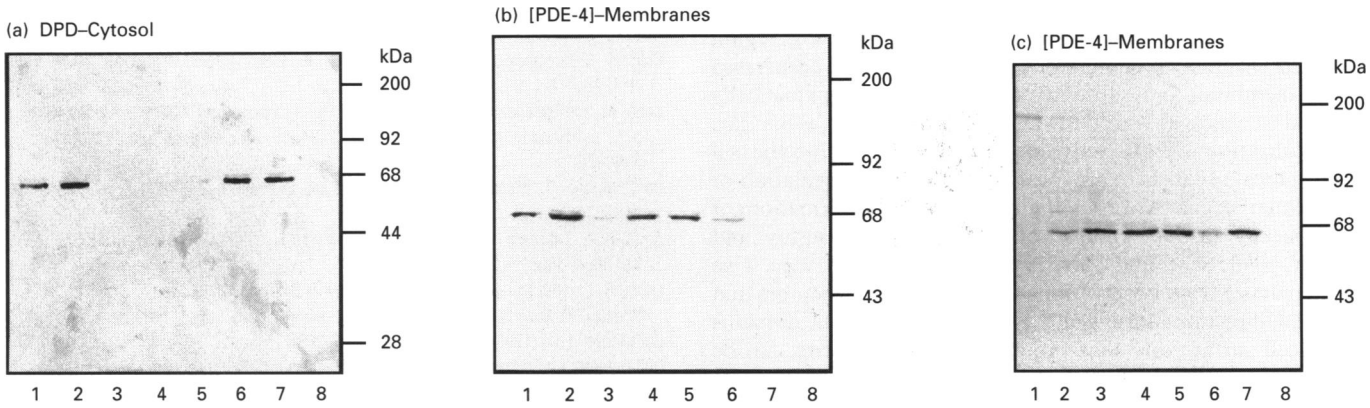

Figure 3 Distribution of immunoreactive DPD and PDE-4 in membrane and cytosol fractions from various brain regions

(**a**) Western blots of cytosol from various brain regions (100 μg of protein/lane) using the DPD/PDE-4 antiserum. (Key to lanes: 1, hypothalamus; 2, cortex; 3, brain stem; 4, cerebellum; 5, mid-brain; 6, striatum; 7, hippocampus; 8, pituitary). (**b**) Western blots of membranes from various brain regions (100 μg of protein/lane) using the DPD/PDE-4 antiserum. (Key to lanes: 1, hypothalamus; 2, cortex; 3, mid-brain; 4, striatum; 5, hippocampus; 6, cerebellum; 7, brain stem; 8, pituitary). (**c**) Western blots of membranes from various brain regions (100 μg of protein/lane) using the PDE-4-specific antiserum. (Key to lanes: 1, brain stem; 2, cerebellum; 3, hippocampus; 4, striatum; 5, cortex; 6, mid-brain; 7, hypothalamus; 8, pituitary). These data show typical experiments of those performed at least three times using different brain preparations and using two sets of antisera. It should be noted that different lane numbers are used for different panels.

identified using the immunoblotting procedure (Table 1). In particular, they confirmed that no PDE-IV$_B$ activity occurred within the pituitary and that the cytosolic fractions from the brain stem, cerebellum and mid-brain were devoid of DPD activity (Table 1). Thus, within specific brain regions we observed the selective expression of PDE-IV$_B$ isoforms.

Fractional activities of DPD and PDE-4

Rolipram acts as a potent, selective inhibitor of type-IV PDEs with K_i values of the order of 0.4–4 μM. In contrast, for other isoforms it either fails to inhibit or does so only very weakly with K_i values > 100 μM (Shahid and Nicholson, 1990; Henkel-Tigges and Davis, 1990; Gordeladze, 1990; Conti et al., 1991; Shakur et al., 1993). Thus the fraction of PDE activity that can be inhibited by low concentrations of rolipram may be taken as an approximation of the amount of type-IV PDE activity present in crude extracts. For these comparative analyses we have routinely used a cyclic AMP substrate concentration of 1 μM. This is at a level which is below the activation threshold for protein kinase A and is of a magnitude seen in a number of isolated cells under resting/basal conditions (see Houslay, 1990). When cyclic AMP levels rise upon activation of adenylate cyclase then the relative activities of various PDEs will change, depending upon their K_m and $V_{max.}$ values. That the type-IV PDEs show similar K_m values, presumably because of their high homology over their catalytic domain (Charbonneau, 1990), makes it likely that their relative activities will stay constant over a range of cyclic AMP concentrations. In contrast, changes in relative activities will be seen with respect to other PDE families, especially if coupled to alterations in intracellular Ca^{2+} and cyclic GMP, which can act to regulate the activity of specific isoforms (Beavo, 1990; Manganiello et al., 1990; Houslay and Kilgour, 1990). These various scenarios have been explored in hepatocytes (Houslay, 1990) where the kinetic properties of cyclic AMP generation and degradation have been fully characterized for the range of enzyme systems involved. The calculation presented here can thus only indicate the possible significance of these particular type-IV$_B$ PDE splice variants by showing their relative magnitudes in various brain regions. This does not take account of the fact that a variety of cell types will be found in these regions which will further complicate the issue as regards relative expression of PDE isoforms and the nature of their regulatory mechanisms.

Using this approach (Table 3), we failed to identify any type-IV PDE activity in the pituitary. This not only confirms the absence of DPD and PDE-4 noted in our studies using antisera but also suggests that the pituitary is devoid of PDE-IV$_A$ -IV$_C$ and -IV$_D$ activities. In the various other brain regions we found a remarkable consistency, with type-IV PDE activity forming some 30–41% of the total cytosolic PDE activity and some 44–64% of the membrane PDE activity (regional variation range; Table 2).

We were able to calculate that DPD activity accounted for 13–35% of the cytosolic type-IV activity and approx. 10% of the total cytosolic PDE activity in the regions where it was expressed (regional variation range; Table 2). Thus DPD, under these conditions, appears to constitute a significant proportion of the total PDE activity in a number of brain regions. However, as DPD has a low K_m for cyclic AMP (Table 3) it is likely that it will be rapidly saturated when adenylate cyclase is activated. Thus any effective regulation through this species would have to be at the level of alterations in enzyme levels, as has been noted for certain type-IV PDEs (Conti et al., 1991; Swinnen et al., 1991) or by some post-translational modification which would alter its $V_{max.}$.

In membranes, PDE-4 activity comprised some 20–30% of the total PDE activity in most brain regions. Exceptions were the pituitary, where it is absent, and the mid-brain, where it accounted for less than 10% of the total (Table 3). It does, however, form (Table 2) a major proportion (40–50%) of the total type-IV PDE activity in all of the regions studied save the mid-brain (approx. 20%) and pituitary.

The PDE-IV$_B$ activity, taken here as representing that of both DPD and PDE-4, is then highest in the brain stem, cortex, hippocampus, hypothalamus and striatum but much lower in the cerebellum and the mid-brain (Table 2). The contribution of PDE-4 and DPD relative to each other is, however, markedly different in various brain areas. For example, PDE-4 provides all of the IV$_B$ activity in brain stem, cerebellum and mid-brain but

only a half to two-thirds of the activity in cortex, hypothalamus and striatum and only a third in hippocampus, the sole region where DPD activity predominates. The reason for the occurrence of both membrane and cytosolic versions of apparently kinetically identical enzymes, as with DPD and PDE-4 (Table 3), is unclear. The localization of PDE activities to membranes within the cell might indicate that there are local controls on the regulation of intracellular cyclic AMP, perhaps through the formation of metabolically distinct 'pools' of cyclic AMP (Houslay and Kilgour, 1990; Scott and Carr, 1992; Brunton et al., 1981). This gains credence from observations that cell-surface receptors and their signalling mechanisms may be confined to specific domains of the cell surface and that protein kinase A-II forms can be targeted to associate with distinct membrane-bound proteins (Scott and Carr, 1992). Membrane association may also confer additional properties on an enzyme, such as susceptibility to regulation and altered stability, as noted for a splice variant of PDE-IV_A, RD1 (Shakur et al., 1993).

These studies provide the first evidence for the expression of native PDE type-IV_B isoforms. These appear to reflect the products of cloned cDNA species for PDE-4 and DPD. Using anti-peptide sera we have shown that they can be identified not only by Western blotting but also by specific immunoprecipitation of active enzymes. That PDE-4 is found exclusively associated with membranes and DPD in the cytosol suggests that the role of the unique N-terminal domain found in PDE-4 is to allow for membrane association. PDE-4 and DPD show distinct distribution patterns in various brain areas, implying differences in gene expression and in mRNA splicing. The molecular mechanisms which regulate the expression of these isoforms, their individual functional roles and the means by which membrane association of PDE-4 occurs remain to be defined. However, PDE-4 and DPD appear to constitute a significant fraction of the PDE activity in brain and their differential expression suggests that alterations in their function and expression, as well as the action of inhibitors which can differentiate between the type-IV_B subtypes, may have selective effects upon brain function.

This work was supported by the Medical Research Council (U.K.), Wellcome Trust and Scottish Home and Health Department (Scotland, U.K.). We are extremely grateful to Dr. M. Wigler (Cold Spring Harbor Laboratories, Cold Spring Harbor, U.S.A.) for his very generous gift of the cDNA for DPD.

REFERENCES

Beavo, J. A. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D., eds.), vol. 2, pp. 3–15, John Wiley & sons, Chichester, New York

Bolger, G. (1994) Cell Signalling, in the press

Bolger, G., Michaeli, T., Martins, T., St. John, T., Steiner, B., Rodgers, L., Riggs, M., Wigler, M. and Ferguson, K. (1993) Mol. Cell. Biol. **13**, 6558–6571

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Brunton, L. L., Hayes, J. S. and Mayer, S. E. (1981) Adv. Cyclic Nucleotide Res. **14**, 391–397

Bushfield, M., Griffiths, S. L., Murphy, G. J., Pyne, N. J., Knowler, J. T., Milligan, G., Parker, P. J., Mollner, S. and Houslay, M. D. (1990) Biochem. J. **271**, 365–372

Charbonneau, H. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D., eds.), vol. 2, pp. 267–298, John Wiley & Sons, Chichester, New York

Chen, C. N., Denome, S. and Davis, R. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9313–9317

Colicelli, J., Birchmeier, C., Michaeli, T., O'Neill, K., Riggs, M. and Wigler, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3599–3603

Conti, M. and Swinnen, J. V. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D. eds.) vol. 2, pp. 243–266, John Wiley & Sons, Chichester, New York

Conti, M., Jin, C., Monaco, L., Repaske, D. R. and Swinnen, J. V. (1991) Endocr. Rev. **12**, 218–234

Davis, R. L., Takayasu, H., Eberwine, M. and Myres, J. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3604–3608

Davis, R. L. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D., eds.) vol. 2, pp. 227–241, John Wiley & Sons, Chichester, New York

Gordeladze, J. O. (1990) Biosci. Rep. **10**, 375–388

Guit-Goffioul, F., Gerard-Vandehove, M. A., Troisfontaines, B., Brelet, M., von Frenckell, R. and Bobon, D. (1987) Acta Psychiatr. Belg. **87**, 396–399

Hebenstreit, G. F., Fellerer, K., Fichte, K., Fischer, G., Geyer, N., Meya, U., Sastre-Hernandez, M., Schony, W., Schratzer, M. and Soukop, W. (1989) Pharmacopsychiatry **22**, 156–160

Henkel-Tigges, J. and Davis, R. L. (1990) Mol. Pharmacol. **37**, 7–10

Houslay, M. D. (1990) Cell. Signal. **2**, 85–98

Houslay, M. D. and Kilgour, E. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D., eds.) vol. 2, pp. 185–226, John Wiley & Sons, Chichester, New York

Jin, S. L. C., Swinnen, J. V. and Conti, M. (1992) J. Biol. Chem. **267**, 18929–18939

Laemmli, U. K. (1970) Nature (London) **222**, 680–682

Lerner, R. A. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3403–3408

Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. **56**, 658–666

Livi, G. P., Kmetz, P., Mchale, M. M., Cieslinski, L. B., Sathe, G. M., Taylor, D. P., Davis, R. L., Torphy, T. J. and Balcarek, J. M. (1990) Mol. Cell. Biol. **10**, 2678–2686

Manganiello, V. C., Smith, C. J., Degerman, E. and Belfrage, P. (1990) in Molecular Pharmacology of Cell Regulation (Beavo, J. A. and Houslay, M. D., eds.) vol. 2, pp. 87–116, John Wiley & Sons, Chichester, New York

Marais, R. M. and Parker, P. J. (1989) Eur. J. Biochem. **182**, 129–137

Marchmont, R. J. and Houslay, M. D. (1980) Biochem. J. **187**, 381–392

Marchmont, R. J., Ayad, S. R. and Houslay, M. D. (1981) Biochem. J. **195**, 645–652

Mchale, M. M., Cieslinski, L. B., Eng, W.-K., Johnson, R. K., Torphy, T. J. and Livi, G. P. (1991) Mol. Pharmacol. **39**, 109–113

McLaughlin, M. M., Cieslinski, L. B., Burman, M., Torphy, T. J. and Livi, G. P. (1993) J. Biol. Chem. **268**, 6470–6476

Monaco, L., Vicini, E. and Conti, M. (1994) J. Biol. Chem. **269**, 347–357

Nemoz, G., Prigent, M., Moueqquit, S., Fougier, O., Macovschi, O. and Oacheco, H. (1985) Biochem. Pharmacol. **34**, 2997–3004

Palmer, T. M., Taberner, N. and Houslay, M. D. (1992) Cell. Signal. **4**, 365–377

Pears, C., Stabel, S., Cazaubon, S. and Parker, P. J. (1992) Biochem. J. **283**, 515–518

Rutten, W. J., Schoot, B. M. and Dupont, J. S. H. (1973) Biochim. Biophys. Acta **315**, 378–383

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

Scott, J. D. and Carr, D. W. (1992) NIPS **7**, 143–148

Shahid, M. and Nicholson, C. D. (1990) Naunyn-Schmiedeberg's Arch. Pharmacol. **342**, 696–705

Shakur, Y., Pryde, J. G. and Houslay, M. D. (1993) Biochem. J. **292**, 677–686

Swinnen, J. V., Joseph, D. R. and Conti, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 5325–5329

Swinnen, J. V., Tsikalas, K. E. and Conti, M. (1991) J. Biol. Chem. **266**, 18370–18377

Tang, E. Y., Parker, P. J., Beattie, J. and Houslay, M. D. (1993) FEBS Lett. **326**, 117–123

Thompson, W. J. and Appleman, M. M. (1971) Biochemistry **10**, 311–316

Thompson, W. J., Epstein, P. M. and Strada, S. J. (1979) Biochemistry **18**, 5228–5237

Wachtel, H. (1983a) Neuropharmacology **22**, 267–272

Wachtel, H. (1983b) J. Pharm. Pharmacol. **35**, 440–444

Received 5 April 1994/2 June 1994; accepted 16 June 1994