0026-895X/01/5901-54–61$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:54–61, 2001

Vol. 59, No. 1
184/875316
Printed in U.S.A.

"cAMP-Specific" Phosphodiesterase Contributes to cGMP Degradation in Cerebellar Cells Exposed to Nitric Oxide

TOMAS C. BELLAMY and JOHN GARTHWAITE

The Wolfson Institute for Biomedical Research, University College London, The Cruciform Building, Gower Street, London, United Kingdom

Received March 28, 2000; accepted September 27, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Nitric oxide (NO) functions as a diffusible messenger in the central nervous system and elsewhere, exerting many of it physiological effects by activating soluble guanylyl cyclase, so increasing cellular cGMP levels. Hydrolysis of cyclic nucleotides is achieved by phosphodiesterases (PDEs) but the enzyme isoforms responsible for degrading cGMP in most cells have not been identified. We have devised a method for quantitatively monitoring the rate of breakdown of cGMP within intact cells and have applied it to rat cerebellar cell suspensions previously stimulated with NO. In contrast to previous findings in cultured cerebellar cells, there was no evidence from the use of selective inhibitors that PDE 1 participated importantly in cGMP hydrolysis. Moreover, procedures expected to increase PDE 1 activity by raising cytosolic Ca^{2+} concentrations (neurotransmitter agonists, Ca^{2+} ionophore) failed to influence cGMP breakdown. Instead, through the use of inhibitors selective for different PDE families, two isoforms were implicated: a "cGMP-specific" PDE (PDE 5), inhibited by sildenafil and zaprinast, and a "cAMP-specific" PDE (PDE 4), inhibited by low concentrations of rolipram and Ro-20–1724 and by milrinone. An explanation is offered for a participation of PDE 4 based on the high estimated intracellular cGMP concentration (~800 μM) and the low affinity of the enzyme for cGMP. In accordance with predictions, recombinant PDE 4 was shown to hydrolyze high cGMP concentrations in a rolipram-sensitive manner. The widespread use of rolipram to test for a specific involvement of cAMP in cellular phenomena must therefore be questioned.

Nitric oxide (NO), formed from L-arginine, serves in many different tissues as an intercellular signaling molecule (Moncada et al., 1991; Garthwaite and Boulton, 1995). Under physiological conditions, the principal transduction pathway used by NO is activation of soluble guanylyl cyclase (sGC), leading to accumulation of cGMP (Hobbs, 1997). The cellular levels of cGMP are regulated not only by the rate of synthesis but also by the rate of breakdown by phosphodiesterase (PDE) enzymes. At least 11 different families of PDE, having varying affinities for cGMP and cAMP and differing pharmacological properties, are known to exist (Beavo, 1995). However, with the exception of platelets (a homogenous cell population) the isoforms hydrolyzing cGMP in cells stimulated by NO are uncertain: most studies have been conducted on tissue homogenates in which PDEs normally located in different cell types or cell compartments are mixed together. Knowledge of the participating isoforms is important for understanding the dynamics of the signal transduction pathways and for providing pharmacological tools to probe those pathways. There is also the potential for the identification of new medicines, as exemplified by sildenafil for erectile dysfunction (Corbin and Francis, 1999) and rolipram for inflammatory disorders (Teixeira et al., 1997).

The central nervous system, and the cerebellum in particular, expresses abundantly the neuronal isoform of NO synthase (nNOS), which is activated by Ca^{2+}/calmodulin (Abu-Soud et al., 1994). A Ca^{2+}/calmodulin-dependent PDE, PDE 1, is also abundant (Yan et al., 1994) and a current hypothesis is that a rise of intracellular Ca^{2+} (as a result of glutamate receptor activation, for example) simultaneously stimulates nNOS activity and PDE 1 activity. In this way, cGMP accumulation in the stimulated cells is inhibited but neighboring cells with low cytosolic Ca^{2+} are free to respond (Mayer et al., 1992). This could act as a device for ensuring that NO acts in a paracrine, rather than autocrine, manner. In principle, such a mechanism would also permit modulation of the NO-cGMP pathway by other signaling systems. These could act on target cells to change cGMP PDE activity by altering intracellular Ca^{2+}, by modifying the levels of the competing PDE 1 substrate (cAMP) or by changing the phosphorylation state of the enzyme (Beavo, 1995).

The present study was aimed at identifying the PDE activities responsible for degrading cellular cGMP after an NO

This work was supported by The Wellcome Trust and a Medical Research Council Studentship (to T.C.B.).

ABBREVIATIONS: NO, nitric oxide; sGC, soluble guanylyl cyclase; PDE, phosphodiesterase; nNOS, neuronal nitric-oxide synthase; DEA, diethylamine; Hb, hemoglobin; ODQ, 1-*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-*I*-one; (1*S*,3*R*)-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine.

stimulus in the cerebellum. To do so, we have developed a method for measuring the rate of cGMP breakdown in living cells and have applied it to freshly isolated cell suspensions from the developing cerebellum. One of the advantages of this preparation for the present purposes is that, despite the cellular heterogeneity, the cGMP response to NO is apparently confined to a single cell type, the astrocytes. The results indicate that PDE 1 is not involved but rather that PDE 5 and, more surprisingly, a "cAMP-specific" PDE (PDE 4) are responsible for cGMP hydrolysis.

Experimental Procedures

Measurement of cGMP and cAMP in Cerebellar Cell Suspensions. Cell suspensions were prepared from 8-day-old rat cerebella as described previously (Garthwaite and Garthwaite, 1987). Pooled cells from 12 to 14 cerebella were divided into populations of 8 million cells (0.4 ml) and incubated at 37°C in a solution containing 130 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 15 mM Tris·HCl, 1.5 mM $CaCl_2$, and 11 mM glucose, pH 7.4). To avoid possible complications arising from endogenous NO production, the NOS inhibitor L-nitroarginine (100 μM) was added at least 1 h before the experiments were begun.

Accumulation of cGMP was evoked by adding the NO donor, diethylamine NO (DEA/NO), at a supramaximal concentration (1 μM). After various time intervals (typically 2 min), hemoglobin (Hb; 10 μM) was added. Hb immediately scavenges free NO, leading to arrest of sGC activity within a few seconds or less (Bellamy et al., 2000). Subsequently, cGMP levels were followed over time by withdrawing aliquots of cells and inactivating them in boiling hypotonic buffer (50 mM Tris, 4 mM EDTA, pH 7.4). cGMP levels were measured by radioimmunoassay. Extracellular cGMP accounts for only 1 to 2% of the total generated and was not deducted from the totals. cAMP was measured using a commercial kit (Amersham Pharmacia Biotech, Bucks, UK).

The PDE inhibitors were dissolved in DMSO and added at a 1:100 dilution to give the required concentration, 10 min before addition of DEA/NO. The solvent itself was without effect on the cGMP response to DEA/NO (not shown). Where neurotransmitter receptor agonists were used, they were dissolved in DMSO or equimolar NaOH (as appropriate), and added immediately before the addition of Hb. Results are given as the mean cyclic nucleotide levels from independently treated cell populations ± S.E.M.

Quantification of the Rate of cGMP Degradation. The decay of cGMP after application of Hb was described by the integrated Michaelis-Menten equation: $V_p t = K_p ln\,(P_o/P_t) + (P_o - P_t)$, where P_o is concentration of cGMP immediately before addition of Hb and P_t is concentration of cGMP at time t. V_p, K_p, and P_o were found by iteration (Fernley, 1974) using Origin v.4.1 software (MicroCal Software, Northampton, MA).

cGMP Degradation by Recombinant PDE 4B. Extracts from BHK cells transfected with human PDE 4B (Bardelle et al., 1999) were a generous gift from Dr. J. Staddon (Eisai London Research Laboratories Ltd., University College, London, UK). Cytosolic extracts (50 μl containing 0.25 mg of protein in 25 mM HEPES, 5 mM EDTA, 1 mM dithiothreitol, 10 μl/ml leupeptin, 50 μg/ml aprotinin, 200 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride) were incubated at 37°C with a solution (5 μl) containing cGMP (0.72–20 mM), $MgSO_4$ (0.5 M), EGTA (1 mM), 1% DMSO, ± rolipram (0.03–100 μM). After various intervals, aliquots were removed and inactivated (as above). Activity was assessed by loss of cGMP.

Materials. 1-*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-*1*-one (ODQ) and (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid [(1*S*,3*R*)-ACPD] were supplied by Tocris Cookson (Bristol, UK). Sildenafil was supplied by the Chemistry Division, Wolfson Institute for Biomedical Research. All other special chemicals were from Sigma-Aldrich (Poole, Dorset, UK).

Results

Measuring PDE Activity in Intact Cells. The technique has been described briefly in a previous study designed to extract the kinetics of sGC in cells (Bellamy et al., 2000). Hence, we begin by providing fuller details of the procedure before using it to characterize the relevant PDE activity.

Addition of the NO donor DEA/NO (1 μM) to the cerebellar cell suspension led to an initial sharp rise in cGMP levels, which then leveled off to give a quasi-plateau after 1 min (Fig. 1a). The hyperbolic shape of the response mainly reflects the kinetics of sGC, namely activation followed by rapid desensitization (Bellamy et al., 2000). When sGC activity was arrested by addition of Hb, cGMP declined progressively (Fig. 1a). At its simplest, the rate of cGMP degradation (v_d) at a given substrate concentration (P) should

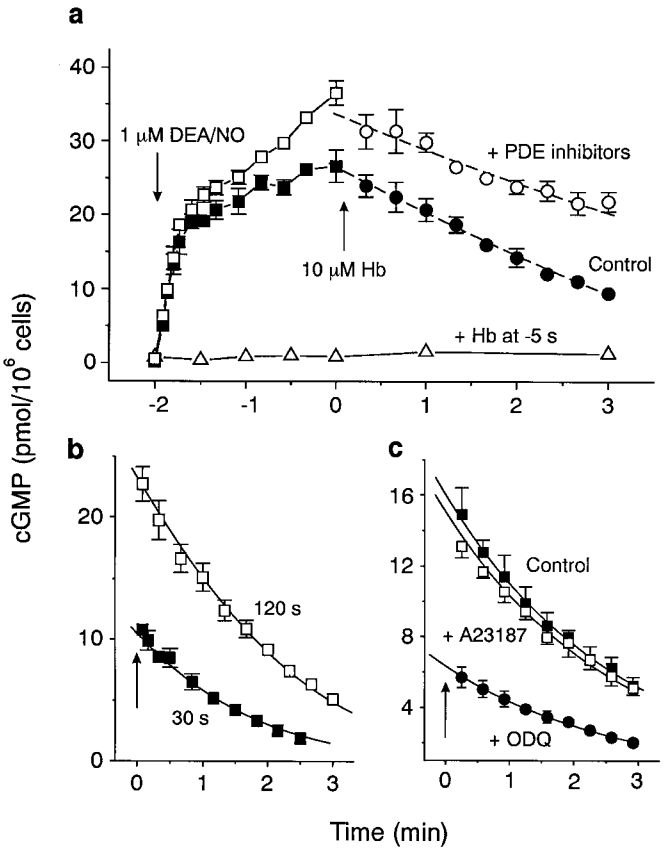

Fig. 1. Time courses of cGMP accumulation and degradation in cerebellar cell suspensions. Shown in a is the accumulation of cGMP following application of 1 μM DEA/NO (□,■) and the subsequent degradation of cGMP after addition of 10 μM Hb (○,●) in the presence (□,○) and absence (■,●) of PDE inhibitors (100 μM zaprinast + 100 μM rolipram). Hb added 5 s before DEA/NO eliminated the cGMP response (△). Data after addition of Hb are fitted to the integrated Michaelis-Menten equation (see *Experimental Procedures*). For control cells (●) V_p = 0.143 pmol/10^6 cells/s, K_p = 8.12 pmol cGMP/10^6 cells, V_p/K_p = 0.0176 s^{-1}. For cells in the presence of PDE inhibitors (○), V_p = 0.143 pmol/10^6 cells/s, K_p = 25.6 pmol cGMP/10^6 cells, V_p/K_p = 0.00559 s^{-1}. In b, the decay curves after addition of Hb after 30 s or 120 s DEA/NO exposure are fitted to common values of V_p (0.305 pmol/10^6 cells/s) and K_p (23.3 pmol/10^6 cells). In c, common values of V_p (2.02 pmol/10^6 cells/s) and K_p (312 pmol/10^6 cells) fit decay curves for different values of P_o (in the presence and absence of 0.3 μM ODQ). Also illustrated is the unchanged rate of cGMP degradation in the presence of A23187 (□). Data are means ± S.E.M. (n = 3–6).

follow Michaelis-Menten-type kinetics according to the equation: $v_d = V_pP / (K_p + P)$. The parameters V_p and K_p are empirical constants describing, respectively, the *apparent* maximum PDE activity and the *apparent* Michaelis constant. The integrated form of the expression (see *Experimental Procedures*) should then describe the fall in cellular cGMP levels over time. As shown in Fig. 1a, the integrated equation provided a good fit to the experimental data. Mean values for the parameters V_p and K_p derived from such fits were 0.81 ± 0.32 pmol/10^6 cells/s and 107 ± 50 pmol/10^6 cells, respectively (n = 9). The rather large standard errors reflect the fact that the absolute values can interchange markedly without having much impact on the net rate of degradation at a given substrate concentration. More revealing is the ratio V_p/K_p, which gives an index of catalytic efficiency, because this ratio cannot vary much without the rate of degradation being significantly affected. Accordingly, there was much less interexperiment variation in the estimations of V_p/K_p, which averaged 0.011 ± 0.001 s^{-1} (n = 9).

The utility of the method depends on cellular PDE activity behaving in a substrate-linked fashion. This was examined in two ways. First, Hb was added after different periods of DEA/NO stimulation (30 s and 120 s), an experiment that tests both for variations in PDE activity over time and for changes in activity at different starting cGMP concentrations. At both time points, subsequent cGMP degradation was well described by the integrated Michaelis-Menten equation using common values of V_p and K_p (Fig. 1b). As a further test, the initial cGMP accumulation was reduced by about 70% using the sGC inhibitor ODQ (0.3 μM). Again, the same values of V_p and K_p fitted the decline in cGMP in control and ODQ-treated cells (Fig. 1c).

Pharmacological Identification of Active PDE Isoforms. Inhibitors selective for different classes of PDE were initially examined using a screening procedure in which cGMP levels were measured 5 min after removal of NO (by addition of Hb). By this time, cGMP in control cells had decayed almost to basal levels. An effective inhibitor of cGMP breakdown, therefore, will cause cGMP to remain elevated relative to that in control cells. The PDE 1-selective inhibitor, nicardipine (300 μM; Agullo and Garcia, 1997), the PDE 2-selective inhibitor, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA; 300 μM; Michie et al., 1996), and the PDE 3-selective inhibitor cilostamide (100 μM; Manganiello et al., 1995), all reduced degradation by only a small extent (Table 1). Another PDE 3-selective inhibitor, milrinone (Manganiello et al., 1995; Schudt et al. 1996), was more effective, causing a graded increase in residual cGMP at concentrations of 30 μM and above (Fig. 2). The PDE 4-selective inhibitor rolipram (Schudt et al. 1996) also caused a concentration-dependent increase in residual cGMP levels (Fig. 2). The maximum effect was similar to that found with milrinone at 300 μM, but the potency of rolipram was much higher (EC_{50} ~30 nM). A second PDE 4-selective inhibitor, Ro-20–1724 (Schudt et al. 1996), gave results very similar to those of rolipram, although its potency was somewhat lower (EC_{50} ~100 nM; Fig. 2). No additional effects of either of the two drugs were observed at concentrations as high as 100 μM (not illustrated).

The PDE 5-selective inhibitor, sildenafil (Turko et al., 1999), was the single most effective agent tested. As the sildenafil concentration was raised over the range 3 to 300 μM, degradation was progressively blocked. At the highest concentration tested (300 μM), the residual cGMP was approximately 10-fold higher than in control cells (Fig. 2). The EC_{50} for sildenafil was about 50 μM. A second PDE 5-selective inhibitor, zaprinast, also increased residual cGMP but the effect was apparently submaximal at the highest concentration tested (300 μM). Judging by the degree of rightward shift of the concentration-response curve, zaprinast was about 30-fold less potent than sildenafil (Fig. 2).

As a control, the compounds sildenafil, rolipram, and zaprinast were examined for possible effects on the level of cGMP in the absence of DEA/NO. After a 10-min incubation, none caused a significant change. The values (pmol cGMP/10^6 cells ± S.E.M.) were: control cells, 0.41 ± 0.08; sildenafil (300 μM), 0.29 ± 0.03; rolipram (1 μM), 0.35 ± 0.07; and zaprinast (300 μM), 0.60 ± 0.04 (n = 3–6).

The two different maxima exhibited by the PDE 4-selective inhibitors and sildenafil suggested that (at least) two discrete isoforms of PDE were operating. This was investigated further by coapplying selected inhibitors with a supramaximal concentration of rolipram (1 μM). With a combination of rolipram and Ro-20–1724 (3 μM) or milrinone (300 μM), the residual cGMP was no different from that found with any of

TABLE 1

Effects of various agents on the level of cGMP remaining in cells 2 or 5 min after removal of NO with Hb

The cGMP level remaining in control cells (see Fig 1a) in each experiment has been subtracted (n = 3–6). The mean residual cGMP in control cells was 6.02 ± 1.33 pmol cGMP/10^6 cells (n = 6) after 2 min and 2.99 ± 0.26 pmol cGMP/10^6 cells (n = 8) after 5 min. Significance was determined by Student's unpaired t test (*P < 0.05; ***P < 0.001).

Compound	Net residual cGMP	
	2 min after Hb	5 min after Hb
	pmol / 10^6 cells	
Glutamate (1 mM)	−0.21 ± 0.17	
Kainate (100 μM)	−0.58 ± 0.64	
(1*S*,3*R*)-ACPD (100 μM)	−0.57 ± 0.26	
Noradrenaline (100 μM)	0.63 ± 0.57	
A23187 (10 μM)	0.30 ± 0.80	
Nicardipine (300 μM)		3.10 ± 0.23***
EHNA (300 μM)		1.44 ± 0.31*
Cilostamide (100 μM)		1.01 ± 0.08***
Sildenafil (300 μM)		19.9 ± 0.34***

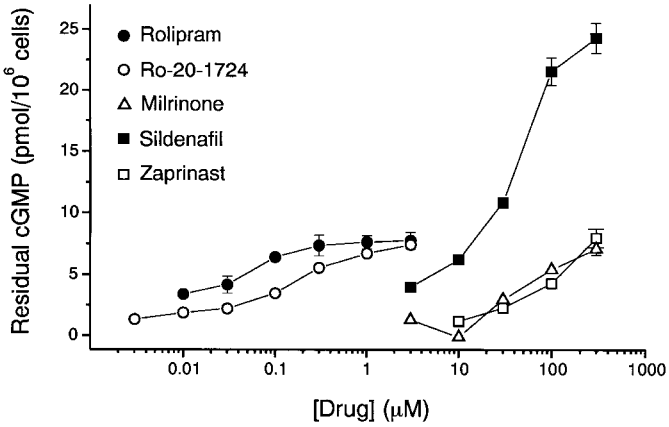

Fig. 2. Concentration-response curves for prevention of cGMP breakdown by PDE inhibitors. Data are the mean levels of residual cGMP 5 min after addition of Hb. In all cases, the corresponding residual cGMP in control cells (mean value, 2.99 ± 0.26 pmol cGMP/10^6 cells; n = 8) has been subtracted.

the compounds individually. In contrast, zaprinast (300 μM) and sildenafil (300 μM) both produced additional inhibition of cGMP degradation (Fig. 3). Furthermore, the combination of sildenafil plus rolipram was more effective than sildenafil alone (Fig. 3), such that 5 min after the Hb addition, cGMP remained at about 70% of its starting value.

The action of selected combinations of inhibitors on cGMP accumulation and on the rate of cGMP degradation was analyzed more thoroughly and quantitatively by examining the time-courses of the rise and fall in cellular cGMP. In the presence of zaprinast and rolipram, there was a progressive increase in the DEA/NO-stimulated cGMP accumulation compared with control cells, the change becoming more marked with time (Fig. 1a). After addition of Hb, cGMP fell more slowly than in control cells. The decay was still smooth and it continued to be well described by the integrated Michaelis-Menten equation. Calculation of the index of PDE catalytic efficiency, K_p/V_p, indicated a 3-fold reduction, from 0.018 s^{-1} to 0.006 s^{-1}. Similar experiments were carried out using sildenafil (100 μM) plus rolipram (1 μM). The overall effects were similar to, but greater than, those seen with zaprinast plus rolipram (see Bellamy et al., 2000). Analysis of the decay phase indicated that K_p/V_p was reduced 4-fold, from 0.011 to 0.003 s^{-1}. Neither the combination of zaprinast plus rolipram (Fig. 1a) nor sildenafil plus rolipram (Bellamy et al., 2000) influenced cGMP levels in the absence of DEA/NO.

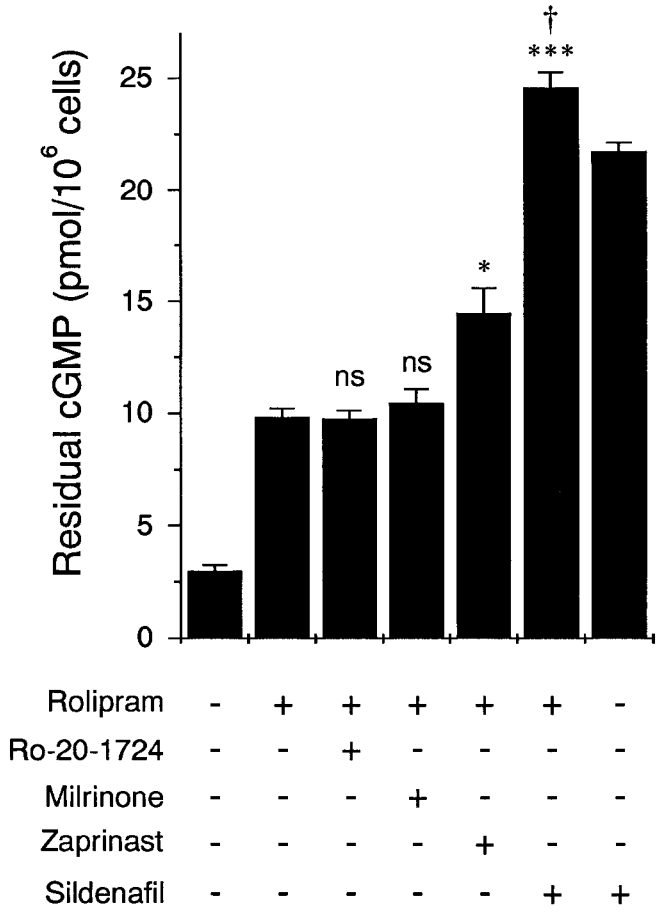

Fig. 3. Effect of combinations of PDE inhibitors. Data are the mean total cGMP levels remaining 5 min after addition of Hb (n = 6–9). *$P < 0.05$; ***$P < 0.001$; ns, not significant, each compared with rolipram alone; †$P < 0.05$ compared with sildenafil alone.

Further Tests for Involvement of PDE 1. The above pharmacological results indicated that PDE 1 does not participate noticeably in cGMP hydrolysis in the cerebellar cells, a finding that conflicts with conclusions based on measurements of PDE activity in homogenates of cultured cerebellar cells (Agullo and Garcia, 1997). One explanation could be that, because Ca^{2+}/calmodulin enhances the activity of PDE 1, it may be necessary to raise Ca^{2+} to observe the enzyme in action. This was tested in two ways. First, neurotransmitter receptor agonists previously shown to raise the cytosolic Ca^{2+} concentration in cerebellar astrocytes (Murphy and Pearce, 1987) were examined for their ability to accelerate the decline in cGMP. For this purpose, the agonists were added immediately before the Hb and cGMP was measured 2 min later, when the level had normally fallen by 50% (Table 1). Neither glutamate itself nor agonists activating selectively the AMPA/kainate or metabotropic glutamate receptors [kainate and (1*S*,3*R*)-ACPD] significantly affected the level of residual cGMP. Noradrenaline was similarly without effect. Moreover, a 1-min pretreatment of the cells with these receptor agonists had no effect on the subsequent time-courses of DEA/NO-stimulated cGMP accumulation (data not shown).

The second method used was to apply the Ca^{2+} ionophore, A23187. This also had no effect on the residual cGMP 2 min after Hb addition (Table 1). As a positive control, A23187 was added before DEA/NO and this led to a subsequent reduction (70%) in the ensuing cGMP accumulation, as predicted, because of uncompetitive inhibition of sGC (not shown; see Parkinson et al., 1999). Furthermore, the concentrations of intracellular Ca^{2+} required to cause this direct inhibition of sGC are severalfold greater than those required for maximal activation of PDE 1 (Agullo and Garcia, 1997), indicating that the magnitude of the Ca^{2+} influx would have been sufficient to reveal any PDE 1-mediated degradation of cGMP. However, conclusions based on measurements at a single time point could be wrong if, in the presence of increased cellular Ca^{2+}, the kinetics of PDE becomes aberrant. Hence, to examine the effect of A23187 more rigorously, the time course of cGMP hydrolysis was followed over 3 min. There was no alteration in the rate of decay compared with control cells throughout the period (Fig. 1c).

Possible Role of cAMP. An explanation for the ability of inhibitors of PDE 4, normally considered a "cAMP-specific" isoform, to attenuate cGMP hydrolysis is that, by inhibiting cAMP breakdown, they might raise the level of cAMP that could, in turn, competitively inhibit cGMP hydrolysis by a PDE able to act on both cyclic nucleotides (i.e., PDEs 1, 2, 3, or 10; Beavo, 1995; Soderling et al., 1999). When measured in the same cells, however, rolipram inhibited cGMP breakdown without altering cAMP levels (Fig. 4). To examine the possibility further, cells were pretreated for 10 min with forskolin (an activator of adenylyl cyclase) before being exposed to DEA/NO and then Hb, as usual. Five minutes later, cAMP was at high levels but there was little or no effect on cGMP breakdown (Fig. 4).

Degradation of cGMP by Recombinant PDE 4B. To examine directly the ability of PDE 4 to degrade cGMP, extracts of BHK cells over-expressing recombinant human PDE 4B were used. cGMP was added at a concentration

estimated to be attained in the cerebellar cells exposed to NO (~800 μM; see *Discussion*). The cGMP concentration declined progressively to the extent that about 85% was degraded after 1 h and this hydrolysis was almost eliminated by 10 μM rolipram (Fig. 5a). A Lineweaver-Burk analysis (Fig. 5a inset) indicated that the K_m value for cGMP was 4.8 mM and that the apparent V_{max} was 24 nmol cGMP/mg of protein/min. For comparison, the same cell extracts hydrolyze cAMP with a K_m value of 5.5 μM (Bardelle et al. 1999) and an apparent V_{max} (calculated from the data of Bardelle et al.) of about 20 nmol/mg of protein/min at 20°C or, assuming a temperature coefficient (Q_{10}) of 2, 68 nmol/mg of protein/min at 37°C. A 3-fold greater maximal activity of PDE 4 against cAMP compared with cGMP accords with an earlier study (Beavo, 1988). The IC_{50} value of rolipram for inhibition of degradation of 800 μM cGMP over 1 h was 0.9 μM (Fig. 5b).

Discussion

Despite extensive reports in the literature concerning phosphodiesterases, quantitative analysis of their activity in intact cells does not seem to have been attempted previously. Monitoring the behavior of the enzymes within their normal environment, however, is essential for understanding their physiological functioning and the possible modulation of their activity by cellular signaling pathways. The cerebellum, used here, has the advantage of low PDE activity, such that cGMP decay can be followed over several minutes. The fact that PDE inhibitors did not reduce the PDE catalytic efficiency index (V_p/K_p) very dramatically (maximally 4-fold) reflects the fact that the index is so low in the first place (0.01 s^{-1}). For comparison, it can be estimated that in human platelets, the uninhibited V_p/K_p is about 100-fold larger (1.4 s^{-1}) and that the appropriate inhibitors (sildenafil plus EHNA) reduce the index 200-fold (Bellamy et al., 2000).

PDEs Degrading cGMP after NO Stimulation of sGC. The inhibitors tested in this study have been well characterized in homogenate assays and against purified PDE subtypes (Table 2). Their use in the cerebellar cells indicates that two PDEs are at work: one inhibited by rolipram, Ro-20–1724, and milrinone and the other inhibited by sildenafil and zaprinast. These properties are those of PDE 4 and PDE 5, respectively. Consistent with this conclusion are the relative inhibitory potencies: rolipram>Ro-20–1724>milrinone is expected for PDE 4, and sildenafil>zaprinast is expected for PDE 5. The PDE families 6,7, and 8 could not easily be examined because of the absence of selective inhibitors. However, the functional and pharmacological properties of these enzymes make them unlikely candidates: PDE 6 activity appears confined to the retina and both PDE 7 and 8 are "cAMP-specific" PDEs that are insensitive to rolipram or Ro-20–1724. Even in the presence of maximal concentrations of sildenafil and rolipram, some cGMP degradation persisted (30% during 5 min). Although this could reflect the activity of another PDE isoform, it may simply be a consequence of the mechanism of action of sildenafil and rolipram: the efficacy of competitive inhibitors such as these will be inherently limited by the associated rise in cGMP concentration.

From other perspectives, the conclusion that PDE 4 and 5 are responsible appears inscrutable. First, PDE 4 is known as a "cAMP specific" isoform. Second, for inhibiting degrada-

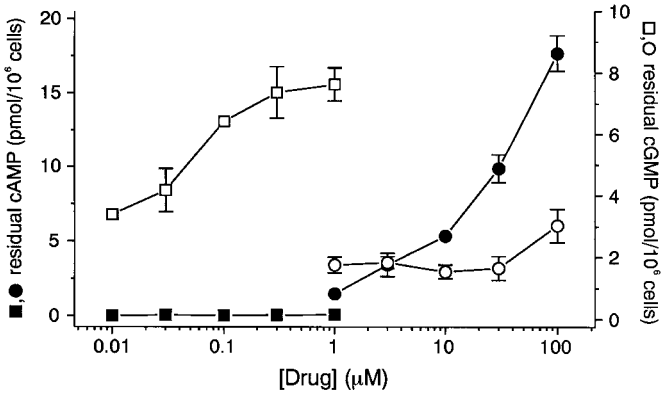

Fig. 4. Concentration-response curves for rolipram (□,■) and forskolin (○,●) on total levels of cGMP (□,○) and cAMP (■,●) remaining 5 min after addition of Hb. Rolipram and forskolin were added 10 min before DEA/NO. Data are means ± S.E.M.; n = 3–6.

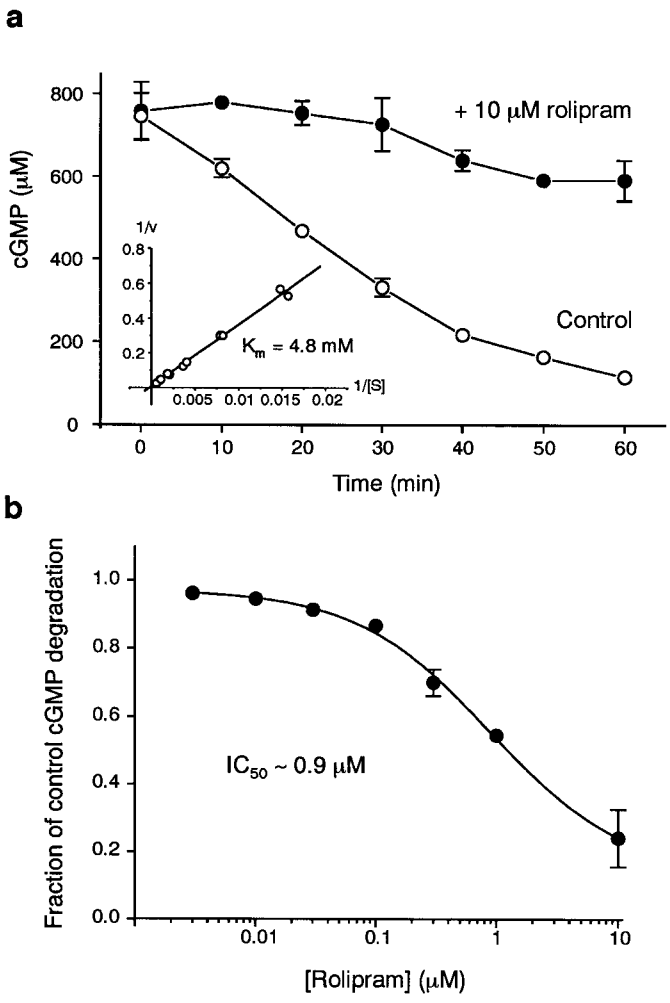

Fig. 5. Degradation of cGMP by recombinant human PDE 4B. In a, a progress curve is illustrated for the decay of cGMP added to a cytosolic extract of BHK cells over-expressing PDE 4B, under control conditions (○) or in the presence of 10 μM rolipram (●) added simultaneously with cGMP. The inset shows a Lineweaver-Burk analysis of degradation of cGMP (serial dilutions from 2000–72.5 μM starting concentration). Rates were determined from the amount of cGMP lost after 20 min. b, concentration-response curve for inhibition by rolipram of cGMP degradation, measured 1 h after addition of 800 μM cGMP. Data are expressed relative to the total cGMP degraded under control conditions (mean ± S.E.M.; n = 3) and are fitted by a logistic curve.

tion of cAMP by PDE 4, rolipram concentrations in the micromolar range are typically used, whereas, in our experiments, nanomolar concentrations of rolipram were effective. Third, sildenafil is a very potent PDE 5 inhibitor, active in low nanomolar concentrations, whereas micromolar concentrations in the tens to hundreds were required in the cerebellar cells; similarly, with zaprinast (active against PDE 5 in submicromolar concentrations), a maximal effect was not evident, even at 300 μM. However, with some quantitative considerations of the prevailing cGMP levels and the application of pharmacological principles, these anomalies become reconcilable.

In cerebellar cell suspensions, NO-evoked cGMP accumulation occurs in the astrocytes (Garthwaite and Garthwaite, 1987; De Vente et al., 1990; Bellamy et al., 2000), which represent about 6% of the total cell number (Cohen et al., 1979) and are approximately spherical with a radius of 5 μm (Garthwaite and Balazs, 1981). Thus, it can be calculated that the 25 pmol of cGMP/10^6 cells formed in response to DEA/NO in the suspension as a whole (Fig. 1a), corresponds to a concentration of about 800 μM in the astrocytes. The potency of any competitive inhibitor (expressed as IC_{50}) depends on the substrate concentration (S) according to the relationship: $IC_{50} = K_i\ (1+ S/K_m)$, where K_i is the inhibitory constant and K_m is the Michaelis constant (Cheng and Prusoff, 1973). Unless $S \ll K_m$, IC_{50} values will reflect the potency of an inhibitor only under particular conditions; the values commonly cited in homogenate studies are derived using cGMP concentrations of 0.5 to 10 μM. At higher substrate concentrations, the IC_{50} value will be higher. From parameters in the literature (Table 2), the predicted IC_{50} of sildenafil for PDE 5 in cells containing 800 μM cGMP is 0.6 to 24 μM, the latter being similar to our estimate of 50 μM. For zaprinast, the predicted IC_{50} is 0.14 to 2.3 mM, which is also consistent with its potency in our experiments.

Although PDE 4 is designated "cAMP specific," the isoform can also metabolize cGMP but with a K_m value well above the concentrations normally used in homogenate assays. Accordingly, we have found that recombinant human PDE 4B is able to degrade cGMP at concentrations calculated to exist in NO-stimulated cells (~800 μM). The initial rate of cGMP hydrolysis by PDE 4B in the BHK cell extracts was about 45 pmol/mg of protein/s (Fig. 5a), which compares with a calculated initial rate of rolipram-sensitive cGMP hydrolysis in the cerebellar astrocytes[1] of 28 pmol/mg of protein/s. Although crude, this comparison supports the feasibility of PDE 4 functioning to degrade cGMP in cells.

The estimated K_m value for PDE 4B of 4.8 mM (Fig. 5) is high compared with another published value for cGMP degradation by PDE 4 (K_m = 310 μM; Beavo, 1988) or with the cGMP concentrations that inhibit cAMP degradation by PDE 4 (approximate K_i = 360 μM; Herman et al., 2000), possibly reflecting differences between PDE 4 isoforms. Collectively, it seems that that the affinity of PDE 4 isoforms for cGMP is in the high micromolar-millimolar range. Hence, at an intracellular cGMP concentration of 800 μM, PDE 4 would be expected to hydrolyze cGMP at about half-maximal rate. Addition of PDE 4-selective inhibitors with K_i values substantially less than the K_m value should then potently inhibit cGMP degradation. For the recombinant PDE 4B, we found an IC_{50} value for rolipram of 0.9 μM (versus 800 μM cGMP). The lower IC_{50} value in the cerebellar cells (30 nM) may reflect accumulation of the drug intracellularly, a different PDE 4 isoform, or intracellular regulatory mechanisms, such as phosphorylation (McPhee et al., 1999).

The PDE 3-selective inhibitor milrinone can also inhibit PDE 4 (K_i = 11 μM) so inhibition of cGMP hydrolysis by PDE 4 could clearly be achieved at the concentrations used in this study. Indeed, the predicted IC_{50} value of 39 μM for milrinone accords well with our experimental data (Fig. 2). Thus, this "PDE 3-selective" drug can inhibit breakdown of cGMP by "cAMP-specific" PDE 4!

Despite initial appearances, therefore, the pharmacological evidence supports the conclusion that the principal cGMP-degrading enzyme in cerebellar astrocytes is PDE 5, with an additional contribution by PDE 4. Other evidence suggests that both these isoforms are expressed in the rat cerebellum, but their precise cellular locations are unclear (Iwahashi et al., 1996; Kotera et al., 1997). In contrast, PDE 1 seems to be the major cGMP-degrading enzyme in homogenates of cultured cerebellar astrocytes (Agullo and Garcia, 1997). Despite exhaustive tests, we could find no obvious role for this enzyme. The discrepancy may be attributable to methodological differences or to differences in PDE expres-

[1] This calculation assumes that, in the cerebellar cell suspension, the initial rate of cGMP degradation is 0.1 pmol/10^6 cells/s (see Fig. 1), that a third of this rate is rolipram-sensitive (see Fig. 3), and that astrocytes make up 6% of the cells (see text) and contain 20 μg cytosolic protein/10^6 cells (based on authors' unpublished observation that 10^6 cells in the cell suspension contain about 50 μg of total protein, of which 40% is cytosolic).

TABLE 2

Literature values of substrate and inhibitor affinities for different PDE families

Values represent the substrate K_m and inhibitor K_i for different PDE families; ranges signify lowest to highest values found.

PDE family	K_m cGMP	K_i Sildenafil	K_i Zaprinast	K_i Rolipram	K_i Milrinone
			μM		
PDE 1	1.2–2.7[a]	0.281[b]	6.65[b]	>1000[c]	48[d]
PDE 2	11[c]	>30[b]	>100[b]		180[d]
PDE 3	0.02–10[d]	16.2[b]	>100[b]		0.26–2.2[d]
PDE 4	310[c]	7.68[b]	77.4[b]	0.06[k]–1.07[l]	11[d]
PDE 5	0.3[c]–5.6[b]	0.0035[b]–0.007[e]	0.13[f]–0.856[b]		~147[c]
PDE 9	0.07[g]–0.17[h]	7[g]	29[g]	>200[g]	
PDE 10	2.9[i]–7.2[j]	>1[i]–2.1[j]	7.1[i]–10.8[j]	47.3[i]	>50.7[j]

[a] Schudt et al., 1996; [b] Ballard et al., 1998; [c] Beavo, 1988; [d] Beavo and Houslay, 1990; [e] Moreland et al., 1998; [f] Turko et al., 1999; [g] Soderling et al., 1998; [h] Fisher et al., 1998; [i] Soderling et al., 1999; [j] Fujishige et al., 1999; [k] Muller et al., 1996; [l] Shakur et al., 1995.

sion between cultured and freshly isolated astrocytes. Our findings indicate that, in these target cells, the downstream effector mechanism for NO signaling is unlikely to undergo modulation by Ca^{2+}-mediated changes in cGMP PDE activity. Whether this applies to other NO targets in the brain remains to be addressed.

From a pharmacological perspective, this study reveals potential pitfalls associated with applying data on PDE activity in homogenates to the regulation of cGMP metabolism within intact cells. In particular, although designated "cAMP specific," PDE 4 could have a direct role to play in regulating cGMP metabolism in cells that accumulate the second messenger to high concentrations. In this respect, it could be argued that the high cGMP concentrations accumulating in the cells over a 2-min exposure to DEA/NO are unphysiological and, hence, that this conclusion is only of academic interest. Examination of Fig. 1a, however, shows that with an exposure to NO of only 4 s, a not unreasonable period from a physiological perspective (Wood and Garthwaite, 1994), cGMP levels are about 25% of maximum. This equates to a cGMP concentration in the astrocytes of around 200 μM, which means (assuming a high micromolar-millimolar K_m range for PDE 4) that significant cGMP hydrolysis would occur through PDE 4. Thus, the phenomenon is of potential physiological significance. Obviously, cGMP concentrations of this magnitude are very high compared with those needed to activate cGMP-dependent protein kinases but cGMP does have other targets. In particular, increasing evidence indicates that cyclic nucleotide-gated ion channels are expressed widely in the brain and other organs (Biel et al., 1994; Kingston et al., 1999; Strijbos et al., 1999; Yao et al., 1999) and, depending on the channel type, cGMP concentrations in the 10 or 100 μM ranges are needed for maximal activity. It is interesting to speculate that these channels represent targets for cGMP, generated in response to NO, in cerebellar astrocytes.

If PDE 4 can contribute to cGMP hydrolysis physiologically, another implication is for the interpretation of results from the many studies that have used rolipram (or other PDE 4 inhibitors) as probes for an involvement of cAMP. As we have demonstrated, low concentrations of rolipram and Ro-20–1724 can have pronounced effects on cGMP degradation. Indeed, with equal concentrations of cGMP and cAMP, a competitive PDE 4 inhibitor will preferentially influence the degradation of cGMP by this enzyme. Furthermore, as exemplified by milrinone, drugs with a weak effect on degradation of cAMP by PDE 4 can have a significant effect on cGMP degradation by the enzyme because of the disparity in affinity (K_m) of PDE 4 for the alternative cyclic nucleotides.

Acknowledgments

We thank the members of Eisai's Research Laboratories in Tsukuba (Japan) and London (UK) for the generous gift of recombinant human PDE 4B. We also thank Dr. D. Madge (Chemistry Division, Wolfson Institute for Biomedical Research) for supplying sildenafil.

References

Abu-Soud HM, Yoho LL and Stuehr DJ (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer. *J Biol Chem* **269:**32047–32050.

Agullo L and Garcia A (1997) Ca^{2+}/calmodulin-dependent cyclic GMP phosphodiesterase activity in granule neurons and astrocytes from rat cerebellum. *Eur J Pharmacol* **323:**119–125.

Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME and Naylor AM (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol* **159:**2164–2171.

Bardelle C, Smales C, Ito M, Nomoto K, Wong EYM, Kato H, Saeki T and Staddon JM (1999) Phosphodiesterase 4 conformers: Preparation of recombinant enzymes and assay for inhibitors. *Anal Biochem* **275:**148–155.

Beavo JA (1988) Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv Second Messenger Phosphoprotein Res* **22:**1–38.

Beavo JA (1995) Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. *Physiol Rev* **75:**725–748.

Beavo JA and Houslay MD (1990) *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation, and Drug Action.* Wiley, New York.

Bellamy TC, Wood J, Goodwin DA and Garthwaite J (2000) Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses. *Proc Natl Acad Sci USA* **97:**2928–2933.

Biel M, Zong X, Distler M, Bosse E, Klugbauer N, Murakami M, Flockerzi V and Hofmann F (1994) Another member of the cyclic nucleotide-gated channel family, expressed in testis, kidney, and heart. *Proc Natl Acad Sci USA* **91:**3505–3509.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Cohen J, Woodhams PL and Balazs R (1979) Preparation of viable astrocytes from the developing cerebellum. *Brain Res* **161:**503–514.

Corbin JD and Francis SH (1999) Cyclic GMP phosphodiesterase-5: Target of sildenafil. *J Biol Chem* **274:**13729–13732.

De Vente J, Bol JGJM, Berkelmans HS, Schipper J and Steinbusch HMW (1990) Immunocytochemistry of cGMP in the cerebellum of the immature, adult, and aged rat: The involvement of nitric oxide. A micropharmacological study. *Eur J Neurosci* **2:**845–862.

Fernley HN (1974) Statistical estimations in enzyme kinetics. The integrated Michaelis equation. *Eur J Biochem* **43:**377–378.

Fisher DA, Smith JF, Pillar JS, St Denis SH and Cheng JB (1998) Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase. *J Biol Chem* **273:**15559–15564.

Fujishige K, Kotera J, Michibata H, Yuasa K, Takebayashi S, Okumura K and Omori K (1999) Cloning and characterization of a novel human phosphodiesterase that hydrolyzes both cAMP and cGMP (PDE10A). *J Biol Chem* **274:**18438–18445.

Garthwaite J and Balazs R (1981) Separation of cell types from the cerebellum and their properties, in *Advances in Cellular Neurobiology*, Vol. 2 (Federoff S and Hertz L eds) pp 461–489, Academic Press, New York.

Garthwaite J and Boulton CL (1995) Nitric oxide signaling in the central nervous system. *Annu Rev Physiol* **57:**683–706.

Garthwaite J and Garthwaite G (1987) Cellular origins of cyclic GMP responses to excitatory amino acid receptor agonists in rat cerebellum in vitro. *J Neurochem* **48:**29–39.

Herman SB, Juilfs DM, Fauman EB, Juneau P and Menetski JP (2000) Analysis of a mutation in phosphodiesterase type 4 that alters both inhibitor activity and nucleotide selectivity. *Mol Pharmacol* **57:**991–999.

Hobbs AJ (1997) Soluble guanylate cyclase: The forgotten sibling. *Trends Pharmacol Sci* **18:**484–491.

Iwahashi Y, Furuyama T, Tano Y, Ishimoto I, Shimomura Y and Inagaki S (1996) Differential distribution of mRNA encoding cAMP-specific phosphodiesterase isoforms in the rat brain. *Mol Brain Res* **38:**14–24.

Kingston PA, Zufall F and Barnstable CJ (1999) Widespread expression of olfactory cyclic nucleotide-gated channel genes in rat brain: Implications for neuronal signalling. *Synapse* **32:**1–12.

Kotera J, Yanaka N, Fujishige K, Imai Y, Akatsuka H, Ishizuka T, Kawashima K and Omori K (1997) Expression of rat cGMP-binding cGMP-specific phosphodiesterase mRNA in Purkinje cell layers during postnatal neuronal development. *Eur J Biochem* **249:**434–442.

Manganiello VC, Taira M, Degerman E and Belfrage P (1995) Type III cGMP-inhibited cyclic nucleotide phosphodiesterases (PDE 3 gene family). *Cell Signal* **7:**445–455

Mayer B, Klatt P, Bohme E and Schmidt K (1992) Regulation of neuronal nitric oxide and cyclic GMP formation by Ca^{2+}. *J Neurochem* **59:**2024–2029.

McPhee I, Yarwood SJ, Scotland G, Huston E, Beard MB, Ross AH, Houslay ES and Houslay MD (1999) Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition. *J Biol Chem* **274:**11796–11810.

Michie AM, Lobban M, Muller T, Harnett MM and Houslay MD (1996) Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: Analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram. *Cell Signal* **8:**97–110

Moncada S, Palmer RM and Higgs EA (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:**109–142.

Moreland RB, Goldstein I and Traish A (1998) Sildenafil, a novel inhibitor of phosphodiesterase type 5 in human corpus cavernosum smooth muscle cells. *Life Sci* **62:**PL309–318.

Muller T, Engels P and Fozard JR (1996) Subtypes of the type 4 cAMP phosphodiesterases: Structure, regulation and selective inhibition. *Trends Pharmacol Sci* **17:**294–298.

Murphy S and Pearce B (1987) Functional receptors for neurotransmitters on astroglial cells. *Neuroscience* **22:**381–394.

Parkinson SJ, Jovanovic A, Jovanovic S, Wagner F, Terzic A and Waldman SA (1999) Regulation of nitric oxide-responsive recombinant soluble guanylyl cyclase by calcium. *Biochemistry* **38:**6441–6448.

Schudt C, Dent G and Rabe KF (1996) *Phosphodiesterase Inhibitors*, Academic Press, London.

Shakur Y, Wilson M, Pooley L, Lobban M, Griffiths SL, Campbell AM, Beattie J, Daly C and Houslay MD (1995) Identification and characterization of the type-IVA cyclic AMP-specific phosphodiesterase RD1 as a membrane-bound protein expressed in cerebellum. *Biochem J* **306:**801–809.

Soderling SH, Bayuga SJ and Beavo JA (1998) Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. *J Biol Chem* **273:**15553–15558.

Soderling SH, Bayuga SJ and Beavo JA (1999) Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A. *Proc Natl Acad Sci USA* **96:**7071–7076.

Strijbos PJ, Pratt GD, Khan S, Charles IG and Garthwaite J (1999) Molecular characterization and in situ localization of a full-length cyclic nucleotide-gated channel in rat brain. *Eur J Neurosci* **11:**4463–4467.

Teixeira MM, Gristwood RW, Cooper N and Hellewell PG (1997) Phosphodiesterase (PDE)4 inhibitors: Anti-inflammatory drugs of the future? *Trends Pharmacol Sci* **18:**164–171.

Turko IV, Ballard SA, Francis SH and Corbin JD (1999) Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol Pharmacol* **56:**124–130.

Wood J and Garthwaite J (1994) Models of the diffusional spread of nitric oxide: Implications for neural nitric oxide signalling and its pharmacological properties. *Neuropharmacology* **33:**1235–1244.

Yan C, Bentley JK, Sonnenburg WK and Beavo JA (1994) Differential expression of the 61 kDa and 63 kDa calmodulin-dependent phosphodiesterases in the mouse brain. *J Neurosci* **14:**973–984.

Yao X, Leung PS, Kwan HY, Wong TP and Fong MW (1999) Rod-type cyclic nucleotide-gated cation channel is expressed in vascular endothelium and vascular smooth muscle cells. *Cardiovasc Res* **41:**282–290.

Send reprint requests to: Professor John Garthwaite, Wolfson Institute for Biomedical Research, University College London, The Cruciform Building, Gower Street, London, WC1E 6BT (E-mail: john.garthwaite@ucl.ac.uk)