0026-895X/91/060711-07$03.00/0

MOLECULAR PHARMACOLOGY, 39:711-717

Phosphodiesterase II, the cGMP-Activatable Cyclic Nucleotide Phosphodiesterase, Regulates Cyclic AMP Metabolism in PC12 Cells

MICHAEL E. WHALIN,[1] JONATHAN G. SCAMMELL, SAMUEL J. STRADA, and W. J. THOMPSON
Department of Pharmacology, University of South Alabama College of Medicine, Mobile, Alabama 36688
Received October 30, 1990; Accepted March 22, 1991

SUMMARY

Analysis of cyclic nucleotide phosphodiesterase (PDE) activity in cellular fractions from cultured rat pheochromocytoma (PC12) cells has shown that the predominant hydrolytic activity in both cytosolic and particulate compartments is characteristic of a PDE II, the cGMP-activatable family of PDE isozymes. Cytosolic PDE activity was purified to a high degree utilizing DE-52 anion exchange and cGMP-Sepharose affinity chromatographies. The physicochemical properties of PC12 PDE II were similar to those of PDE II isolated from particulate or soluble fractions of other tissues, including subunit molecular weight of ~102,000, activation of cAMP hydrolysis by cGMP, and positive cooperative kinetic behavior for cAMP and cGMP hydrolysis. The potential role of PDE II in regulating cAMP metabolism in intact PC12 cells was studied using an [^{3}H]adenine prelabeling technique. Stimulation of PC12 cell adenosine receptors resulted in a 5–8-fold increase in cAMP accumulation. Removal of the adenosine stimulus by the addition of exogenous adenosine deaminase resulted in a rapid decay of cAMP to prestimulated basal levels within 2 min. Treatment of PC12 cells with atrial natriuretic factor or sodium nitroprusside caused 1) increased intracellular cGMP levels, 2) attenuation of adenosine-stimulated cAMP accumulation, and 3) increased rates of cAMP decay after removal of the adenosine stimulus. Treatment of PC12 cells with HL-725 (a potent inhibitor of isolated PDE II activity *in vitro*) caused 1) increased basal cAMP accumulation, 2) potentiation of adenosine-stimulated cAMP accumulation, and 3) retardation of the rate of cAMP decay after removal of the adenosine stimulus. HL-725 blocked both the attenuation of cAMP accumulation and the accelerated rate of cAMP decay observed with the cGMP-elevating agents. These results suggest that, in PC12 cells, drugs or hormones that inhibit PDE II or increase intracellular cGMP levels to activate PDE II can modulate cAMP metabolism by altering the catalytic status of the enzyme.

The rat adrenal medullary pheochromocytoma PC12 cell line is a well characterized cell strain that has been utilized as a model system for the study of neurotransmitter release (1). The cells contain adenosine receptors that are coupled to cAMP synthesis (2, 3) and ANF receptors (4) that are coupled to cGMP synthesis (5).

Several reports have provided evidence that changes in cAMP and cGMP levels are associated with potentiation and attenuation of neurotransmitter release from PC12 cells. For example, Rabe and co-workers (6, 7) showed that an increase in cAMP levels, elicited by adenosine or forskolin, potentiated depolarization-dependent norepinephrine and acetylcholine release from PC12 cells. In other studies, Drewett *et al.* (8) showed that ANF inhibited depolarization-dependent norepinephrine and dopamine release from PC12 cells. The inhibition of release was correlated with a dose-related increase in cGMP levels (9). In the studies described above, cyclic nucleotides appear to play a modulatory role, because they do not affect neurosecretion alone.

Elucidation of the role of cyclic nucleotides in the modulation of cellular processes in PC12 cells is still in a descriptive phenomenological stage. Although it is clear from the evidence that changes in cyclic nucleotide levels are associated in some manner with neurotransmitter release, the molecular mechanisms by which they act have not been elucidated. Whatever role cyclic nucleotides play in modulation of PC12 cell neurosecretion, the regulation of cAMP and cGMP levels can be viewed as an important determinant in their action.

The cyclic nucleotide PDE enzyme system (EC 3.1.4.17) is the only known catabolic pathway for the elimination of cAMP

This work was supported by National Institutes of Health Grants GM 33538 (W.J.T. and S.J.S.) and DK 40593 (J.G.S.) and a grant from the Eli Lilly Co. M.E.W. was supported by a predoctoral fellowship from the Eli Lilly Co. J.G.S. was supported by a Faculty Development Award in Basic Pharmacology from the Pharmaceutical Manufactures Association, Inc.
[1] Present address: Fidia-Georgetown Institute for the Neurosciences, 3900 Reservoir Road N.W., Washington, DC 20007.

ABBREVIATIONS: ANF, atrial natriuretic factor; PDE, cyclic nucleotide phosphodiesterase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SNP, sodium nitroprusside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; HEPES-BS, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered saline.

and cGMP. This family of enzymes represents a fundamental regulatory component modulating the intensity and duration of cellular responses elicited by hormones and neurotransmitters that utilize cyclic nucleotide second messengers. Multiple forms of PDE, with distinct physical, catalytic, immunological, and regulatory properties, are present in mammalian tissues (10, 11). One isoform that is abundant in brain (12, 13) and adrenal gland (14) is PDE II. This isozyme family is unique because cGMP is capable of enhancing the hydrolysis of cAMP (15). PDE II may represent an important component in a regulatory pathway by which cGMP can modulate cAMP metabolism and cAMP-mediated events in cells.

To date, there have been few reports directly related to cyclic nucleotide metabolism in PC12 cells. The present study was undertaken to characterize the PDE activities present in PC12 cells and to examine the potential role of PDE (in particular PDE II) in the regulation of cAMP metabolism in intact PC12 cells. A preliminary report of this research has been presented elsewhere (16).

Experimental Procedures

Materials. All chemicals were reagent grade or better. [2,8-^{3}H]cAMP (specific activity, 36 Ci/mmol) and [8-^{3}H]cGMP (specific activity, 19 Ci/mmol) were purchased from ICN. The tritiated cyclic nucleotides were purified by Dowex 1-X8 (200–400 mesh) chromatography before use (17). [2,8-^{3}H]Adenine (specific activity, 20.7 Ci/mmol) and [8-^{14}C]cAMP (specific activity, 42.4 mCi/mmol) were purchased from New England Nuclear. Dowex 1-X8 (200–400 mesh) resin was obtained from Aldrich, and AG50W-X4 (200–400 mesh, hydrogen form) cation exchange resin was purchased from Bio-Rad Laboratories and treated according to the manufacturer's specifications. Alumina (WN-3), adenosine deaminase (type IV), poly-L-lysine (molecular weight 300,000), and SNP were from Sigma Chemical Co. Adenosine analogs were purchased from Research Biochemicals, Inc., ANF 8–33 (rat) was purchased from Peninsula Laboratories. Dulbecco's modified Eagle's medium was from GIBCO Laboratories; horse and fetal bovine serum were purchased from Hyclone.

Drugs. PDE inhibitors were generously supplied as follows: papaverine [6,7-dimethoxy-1-(3′,4′-dimethoxybenzyl)isoquinoline hydrochloride] and indolidan (LY 195115) [1,3-dihydro-3,3-dimethyl-5-(1,4,5,6-tetrahydro-6-oxo-3-pyridazinyl)-2*H*-indol-2-one], Dr. David Robertson, Eli Lilly and Co.; rolipram (ZK 62711) [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone], Dr. Ian Williams, Pfizer Laboratories; trequinsin (HL-725) [2,3,6,7-tetrahydro-9,10-dimethoxy-3-methyl-2-[2,4,6-(trimethylphenyl)imino]-4*H*-pyrimido[6,1-*a*]isoquinolin-4-one hydrochloride], Hoechst-Roussel Pharmaceuticals, Inc.; CGS 9343B [1,3-dihydro-1-[1-[4-methyl-4*H*,6*H*-pyrrolo[1,2-*a*][4,1]-benzoxaze-pin-4-yl-methyl]-4-piperidinyl]-2*H*-benzimidazol-2-one (1:1) maleate], Dr. Jon Norman, Ciba-Geigy Corporation; Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], Dr. Herbert Sheppard, Hoffmann LaRoche; and zaprinast (M&B 22948) [2-*o*-prooxyphenyl-8-azapurinone], Dr. John Souness May and Baker Co. All drugs and adenosine were dissolved in 100% dimethylsulfoxide.

Cell culture. PC12 cells, provided by Dr. Edward Hawrot (Department of Pharmacology, Yale University School of Medicine), were routinely grown at 37° in a humidified atmosphere of 95% air/5% CO_2, in 75-mm^2 flasks, in Dulbecco's modified Eagle's medium supplemented with 12.5% horse serum, 5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin and were subcultured weekly. For intact cell cAMP metabolism experiments, cells were subcultured into poly-L-lysine-coated six-well (35-mm-diameter wells) dishes at 2.5 × 10^5 cells/well.

PDE activity assay. PDE activity was determined using the modified two-step radioisotopic procedure of Thompson *et al.* (18). Enzyme assays were optimized so that the activity of each PDE isoform could be determined. Incubation mixtures (0.4 ml) contained 40 mM Tris·HCl (pH 7.4–8.0), 10 mM magnesium acetate, 0.2–3.75 mM 2-mercaptoethanol, 30 μg of fatty acid-poor bovine serum albumin, 0.125–1000 μM [^{3}H]cAMP or [^{3}H]cGMP (approximately 100,000 cpm/assay), and cell fraction or purified enzyme giving less than 20% hydrolysis (17). All enzyme assays were performed at 30°.

Purification of PDE II from PC12 cells. Frozen PC12 cells were sonicated (three 10-sec bursts; low setting) in 10 volumes of buffer consisting of 20 mM Tris·HCl (pH 7.5), 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 15 mM benzamidine (buffer A). The cell sonicate was centrifuged at 30,000 × *g* for 30 min at 4°. The resulting supernatant was applied to a 1.6- × 20-cm column containing 5 ml of DEAE-cellulose (DE-52), at a flow rate of 1 ml/min. After sample application, the column was washed with 10 bed volumes of buffer A. PDE activity was eluted with 60 ml of buffer A, using a 0–1 M NaCl gradient at 1 ml/min. Column fractions (1 ml) were assayed at 5 μM [^{3}H]cAMP substrate in the absence and presence of 2 μM cGMP and at 25 μM [^{3}H]cGMP substrate. Peak fractions of cGMP-stimulated cAMP activity were pooled and applied to a 1.6- × 20-cm column containing 10 ml of cGMP-Sepharose affinity matrix that had been prepared by a slightly modified procedure of Martins *et al.* (14) and previously equilibrated with 20 mM Tris·HCl (pH 7.0), 2 mM dithiothreitol, 1 mM EDTA, 15 mM benzamidine (buffer B). After sample application, the column was washed with 50 bed volumes of buffer B containing 500 mM NaCl, followed by 2 bed volumes of buffer B containing 125 mM NaCl. PDE II activity was eluted with 3 bed volumes of buffer B containing 125 mM NaCl and 10 mM cGMP at 23°. The eluate from the cGMP-Sepharose affinity column was concentrated 10–100-fold, using fast-flow granulated hydroxylapatite (12) to remove cGMP and Amicon ultrafiltration.

PAGE. Slab PAGE was performed with 5% stacking and 7.5% separating gels, in 0.1% SDS, according to the method of Laemmli (19). Gels were stained for protein using the silver staining method of Wray *et al.* (20) or by 0.25% Coomassie R-250 in 45% methanol and 10% acetic acid.

Measurement of cyclic AMP accumulation and decay. Intact PC12 cell cAMP accumulation and decay were determined as described by Barber *et al.* (21) and Whalin *et al.* (16, 22). PC12 cells (1 × 10^6 cells/well) in six-well plates were incubated with [^{3}H]adenine (10 μCi/well) for 60 min at 37°, in a humidified atmosphere. Excess extracellular radioactivity was removed, and the cells were washed two times (1 ml each) with HEPES-BS (120 mM NaCl, 4.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.1 mM EDTA, 10 mM glucose, 25 mM HEPES, pH 7.4). The cells were incubated at 37° for 15 min in 1 ml of HEPES-BS before addition of agents. Time courses for adenosine-stimulated cAMP accumulation were generated by incubation of cells with 50 μM adenosine for various times, followed by aspiration of the contents of the wells and addition of 1 ml of ice-cold 5% TCA, containing [^{14}C]cAMP as an internal standard to monitor recovery. The extracted cellular [^{3}H]cAMP was purified using Dowex/alumina double-column chromatography (23). Cyclic AMP values were expressed as percentage of conversion from total ^{3}H, which is >99% ATP (22). For cAMP decay experiments, washed, [^{3}H]adenine-labeled PC12 cells were incubated for 5 min with adenosine (50 μM). Time courses of cAMP decay were initiated by aspiration of the contents of each well, followed immediately by the addition of 0.55 unit of adenosine deaminase in the presence of the indicated agents in 1 ml of HEPES-BS. Cyclic AMP decay was terminated after various time intervals by the aspiration of the media of each well, followed immediately by the addition of 1 ml of ice-cold 5% TCA/[^{14}C]cAMP. Cyclic AMP was isolated and expressed as percentage of conversion or as its natural logarithm, as described above (22).

Miscellaneous. Cyclic GMP levels were determined using radioimmunoassay, as described by Brooker *et al.* (24). Protein was quantitated using the Coomassie dye binding procedure of Bradford (25), with bovine serum albumin as the standard. IC_{50} values were calculated using the program CURVEFIT (26). Kinetic parameters were calcu-

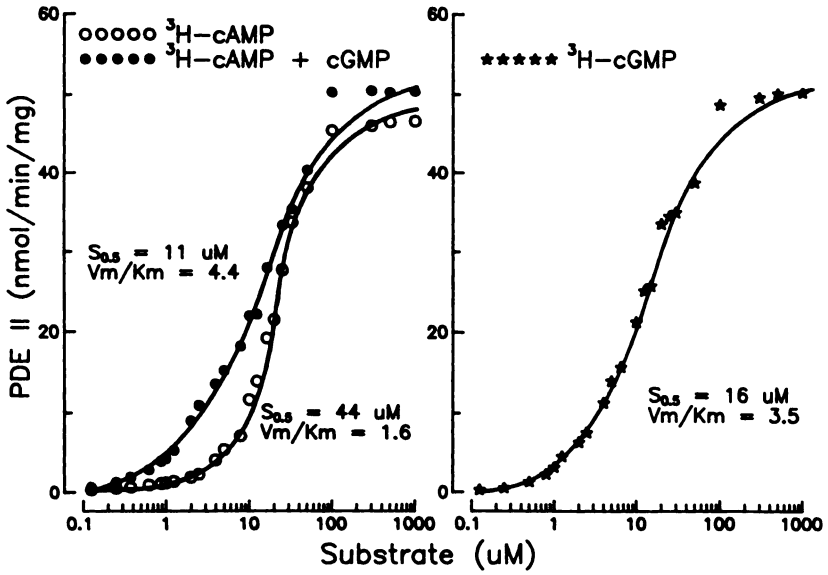

Fig. 1. Effect of cGMP on cAMP hydrolysis in PC12 cell cytosolic fractions. PDE activity was determined using 0.125–1000 μM [^{3}H]cAMP substrate in the absence or presence of 3 μM cGMP (*left*), as described in Experimental Procedures. *Right*, [^{3}H]cGMP hydrolysis using 0.125–1000 μM substrate concentrations.

TABLE 1

Purification of PDE II from PC12 cells

PDE II was purified as described in Experimental Procedures. Total and specific activities of the fractions were determined using 40 μM [^{3}H]cGMP substrate. Fold purification and yield are calculated from the 30,000 × *g* supernatant.

Fraction	Total activity	Specific activity	Fold purification (yield)
	nmol/min	*μmol/min/mg*	
30,000 × *g* supernatant	504	0.0043	
DE-52	267	0.0091	2.1 (53%)
cGMP-Sepharose	16	7.8	1814 (3%)

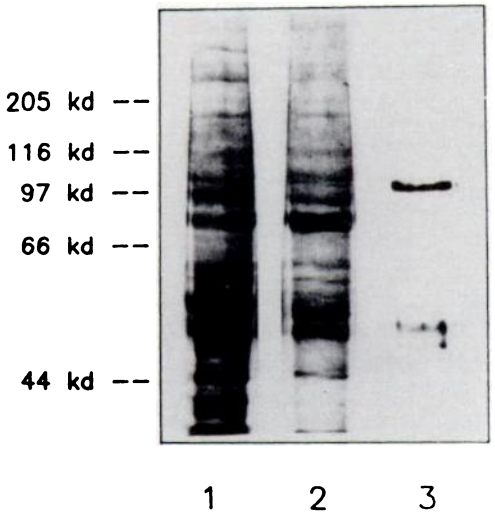

Fig. 2. SDS-PAGE of PC12 type II PDE purification steps. Samples from PC12 fractions were analyzed by 7.5% SDS-PAGE. *Lane 1*, 30,000 × *g* supernatant (9.5 μg); *lane 2*, pooled DE-52 activity fractions (6.3 μg); *lane 3*, cGMP-Sepharose 10 mM cGMP eluate (~1 μg). Proteins in lanes 1 and 2 were visualized by Coomassie staining and those in lane 3 by silver staining, as described in Experimental Procedures.

lated using the program ENZFITTER (version 1.05, 1987), obtained from Elsevier-Biosoft.

Results

Analysis of PDE activity in PC12 cells. Initial experiments were performed in order to determine the relative distribution of cAMP and cGMP hydrolytic activity in cellular fractions prepared from PC12 cells. PDE activity, measured using 100 μM cAMP and cGMP, was distributed equally between particulate and cytosolic compartments (data not shown). Further analysis was conducted under conditions designed to optimize the contributions of various PDE isozymes. In PC12 cell sonicates, 30,000 × *g* supernatant, and pellet fractions, cAMP hydrolysis at 0.25 μM substrate was increased by 3 μM cGMP from 393 to 1416, from 252 to 784, and from 184 to 700 pmol/min respectively). Hydrolysis at 5 μM cAMP substrate was also enhanced by cGMP (data not shown). Maximum activation (473%) was observed at 3 μM cGMP (range, 0.02 to 10 μM) and 2 μM cAMP substrate (range, 0.125 to 20 μM), with varying fold stimulation at different cAMP concentrations. The effect of cGMP on cAMP hydrolysis by PDE in PC12 cells was very similar to that observed with purified PDE II from rabbit and bovine brain particulate fractions (12, 13) and bovine heart, adrenal, and liver cytosolic fractions (14, 27). No PDE I (calcium/calmodulin-activatable) activity was detected in any fraction from PC12 cells.

The effect of cGMP on the kinetics of cAMP hydrolysis in PC12 cytosol was to enhance hydrolysis at substrate concentrations below the $S_{0.5}$ (44 μM) of the enzyme (Fig. 1). The maximum velocity for cAMP hydrolysis was not appreciably different in the absence or presence of cGMP. Eadie-Hofstee

TABLE 2

Inhibitor sensitivity of DE-52 PD activity peak

PDE activity analyses were performed using 2 μM cAMP substrate in the absence or presence of 3 μM cGMP and using 2 μM cGMP substrate. Inhibitor concentrations ranged from 0.1 to 500 μM. IC_{50} values were determined using CURVEFIT analysis. Percentage error (values given in parentheses) is that of the 95% confidence limits at the IC_{50}.

Inhibitor	IC_{50}		
	cAMP	cAMP + cGMP	cGMP
		μM	
Rolipram	>100	>100	>100
Ro 20-1724	>100	>100	>100
Indolidan	>100	>100	>100
CGS 9343B	>100	>100	>100
M & B 22948	>100	>100	>100
HL-725	2.2 (6.2%)	0.54 (9.4%)	0.64 (4.3%)

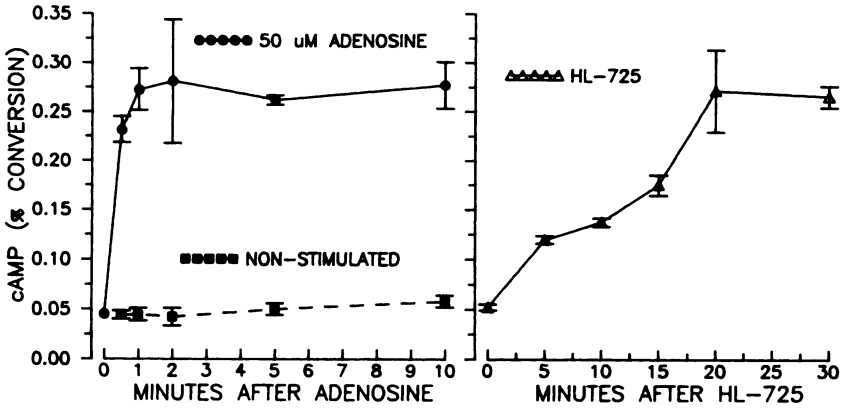

Fig. 3. Time courses of adenosine- and HL-725-stimulated cAMP accumulation in PC12 cells. PC12 cells (1×10^6 cells/well) were prelabeled with [^{3}H]adenine and incubated with or without 50 μM adenosine (*left*) or with 100 μM HL-725 (*right*) for the indicated times. Cyclic AMP was extracted with 5% TCA and purified by Dowex/alumina chromatography, as described in Experimental Procedures. Values represent the mean ± the standard deviation of triplicate determinations.

plots showed positive cooperativity for both cAMP and cGMP hydrolysis (data not shown). Cyclic GMP linearized the cAMP kinetic profile, as has been observed for other PDE II activities. The kinetic parameters of cytosolic PDE II activity from PC12 cells are shown on Fig. 1.

Purification of PDE II from PC12 cells. We have shown previously, using DEAE-cellulose (DE-52) chromatography of PC12 cytosolic fractions, that cAMP, cGMP-stimulated cAMP, and cGMP hydrolytic activities co-elute in a single peak of activity (16). The DE-52 fraction was purified further using cGMP-Sepharose affinity chromatography, as described in Experimental Procedures. A typical purification procedure, using 2 g (wet weight) of PC12 cells, is shown in Table 1, with 7% SDS-PAGE analysis of the steps of purification being shown in Fig. 2. The PDE II of this preparation showed an 1800-fold increase in specific activity from the 30,000 × *g* cytosolic fraction and a yield of 3.1%. The specific activity (at 40 μM cGMP) of the cGMP-eluted activity was ~8 μmol/min/mg. The cGMP-Sepharose eluate (Fig. 2, *lane 3*) showed a major protein band near 102 kDa and minor bands in the 42-kDa range, by silver-stained 7.5% SDS-PAGE. The activities in the major peak from DE-52 (Table 2) and the highly purified enzyme were potently inhibited by the isoquinoline compound HL-725, whereas PDE activity in this fraction was not inhibited by up to 100 μM concentrations of known inhibitors of other PDE

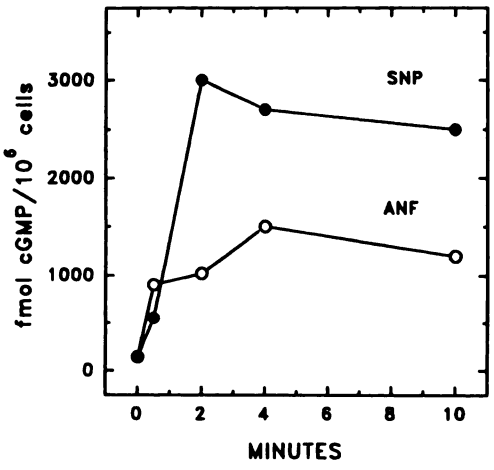

Fig. 4. Effect of ANF and SNP on cGMP levels in PC12 cells. PC12 cells (1×10^6 cells/well) were incubated for the indicated time intervals with 1 μM ANF or 500 μM SNP. Cyclic GMP was extracted from the cells with 5% TCA and quantitated by radioimmunoassay, as described in Experimental Procedures.

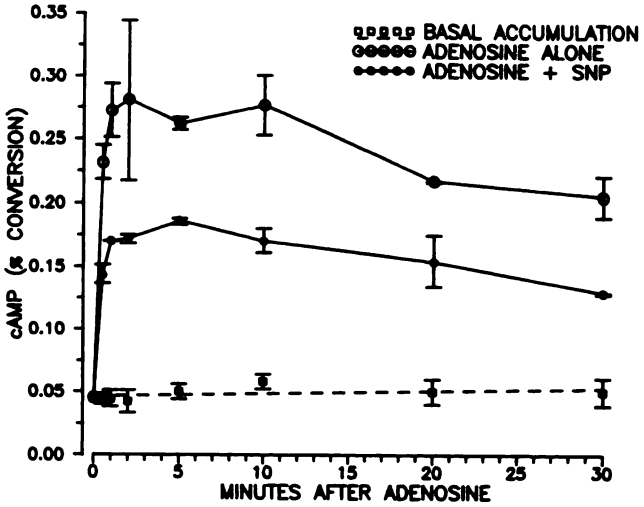

Fig. 5. Effect of SNP on adenosine-stimulated cAMP accumulation in PC12 cells. PC12 cells were prelabeled using [^{3}H]adenine, as described in Experimental Procedures. The cells were preincubated for 10 min in the absence or presence of 500 μM SNP before the addition of 50 μM adenosine to induce accumulation. Basal (nonstimulated) cAMP accumulation is also indicated. Values represent the mean ± the standard deviation from triplicate determinations.

isoforms. 8-Bromo-cGMP or dibutyryl-cGMP had no effect on cAMP hydrolysis at concentrations up to 50 μM (data not shown).

Adenosine-stimulated cAMP accumulation and the effects of PDE inhibitors. The presence of adenosine receptors on PC12 cells has been well documented (2, 3, 7, 28, 29). In agreement with other reports, cAMP accumulation increased very rapidly in response to 50 μM adenosine (Fig. 3, *left*). Cyclic AMP accumulation in response to adenosine reached steady state levels 5–8-fold higher than basal levels in 2–5 min and remained constant for at least 10 min in the continued presence of the agonist. We have shown previously that the isoquinoline derivatives HL-725 and papaverine potentiate cAMP accumulation in response to adenosine (16). The effect of HL-725 was most marked, inasmuch as it showed a >50-fold increase in cAMP levels. Papaverine, which has an approximately 10 fold higher IC_{50} than HL-725, was much less effective. Inhibitors of other isozyme families of PDE, including CGS 9343B (PDE I, calcium/calmodulin-stimulated PDE) (30), indolidan (PDE III, cGMP-inhibitable cAMP PDE) (31), rolipram and RO 20-1724 (PDE IV, cAMP-specific PDE) (32–34), and zaprinast (PDE

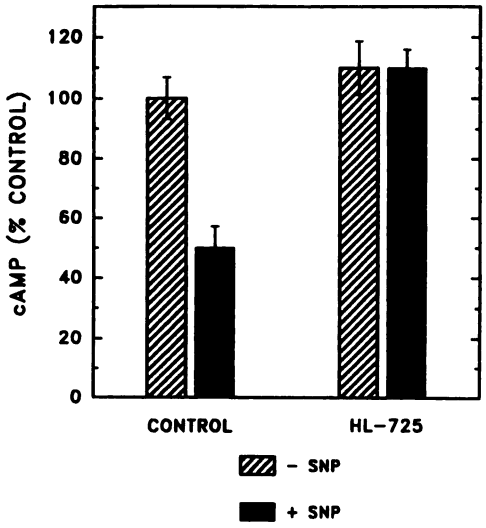

Fig. 6. Effect of HL-725 on adenosine-stimulated cAMP accumulation in the absence or presence of SNP. Prelabeled PC12 cells (1×10^6 cells/well) were incubated for 10 min in the absence or presence of 500 μM SNP, each with or without HL-725 (100 μM). Adenosine (50 μM) was added for 5 min, and cAMP accumulation was analyzed as described in Experimental Procedures. Values represent the mean ± the standard deviation from triplicate determinations.

V, cGMP PDE) (35, 36), did not potentiate adenosine-stimulated cAMP accumulation in PC12 cells (16).

Basal (nonstimulated) cAMP accumulation in PC12 cells was also increased in the presence of 100 μM HL-725. Preincubation with HL-725 required approximately 20 min to reach maximum effect (Fig. 3, *right*). Additionally, HL-725 enhanced cAMP accumulation in PC12 cells induced by 50 μM concentrations of the adenosine analogs (R-)N^6-(2-phenylisopropyl)adenosine (PIA), (S′-*N*-ethylcarboxamidoadenosine) (NECA) (N^6-cyclohexyladenosine)(CHA), (N^6-cyclopentyladenosine) (CPA) (data not shown).

Effect of ANF, SNP, and cGMP analogs on cAMP accumulation. ANF and SNP have been shown to increase cGMP levels in many tissues and cells; data shown in Fig. 4 using 1 μM ANF and 500 μM SNP confirm these findings in PC12 cells (5, 9). These agents activate particulate and soluble guanylate cyclases, respectively (37, 38). SNP showed an approximately 3-fold greater response than ANF, when both agents were tested at maximally stimulating concentrations.

If PDE II is involved in regulating cAMP accumulation in PC12 cells, then activation of the enzyme by increasing cGMP should result in an attenuation of cAMP accumulation. Treatment of PC12 cells with 500 μM SNP (Fig. 5) resulted in an attenuation of the adenosine-stimulated cAMP accumulation. ANF (1 μM) treatment had a similar effect, using 1, 10, or 100 μM adenosine concentrations (data not shown). Data published elsewhere (16) have shown that treatment of PC12 cells with either of two membrane-permeable cGMP analogs, 8-bromo-cGMP or $N^2$2′-*O*-dibutyryl-cGMP, does not attenuate adenosine-stimulated cAMP accumulation.

HL-725 blocked SNP attenuation of adenosine-stimulated cAMP accumulation (Fig. 6). Cyclic GMP-dependent protein kinase, a known cGMP receptor, apparently is not the cellular site for cGMP action in PC12 cells, because the action of 8-bromo-cGMP, a potent activator of the kinase (39), did not mimic the response elicited by ANF or SNP (16). In addition, no measurable immunoreactive cGMP-dependent protein kinase was detectable in PC12 cells by Western blot analysis (data not shown).

Regulation of cAMP decay. Fig. 7 shows that SNP (Fig. 7, *left*) and ANF (Fig. 7, *right*) both increase the slope of cAMP decay curves after termination of the adenosine response with adenosine deaminase. These findings suggest that ANF- and SNP-induced increases in cGMP levels result in an increased rate of cAMP decay by the activation of PDE II, rather than inhibition of adenylate cyclase.

HL-725 inhibits the rate of cAMP decay both in the presence and in the absence of ANF attenuation of adenosine-stimulated accumulation (Fig. 8). The data were recalculated and replotted from those shown in a preliminary report (16) to emphasize the inhibition of cAMP decay rates in response to the drug. These findings indicate that an HL-725-sensitive enzyme is involved in regulating cAMP decay in PC12 cells.

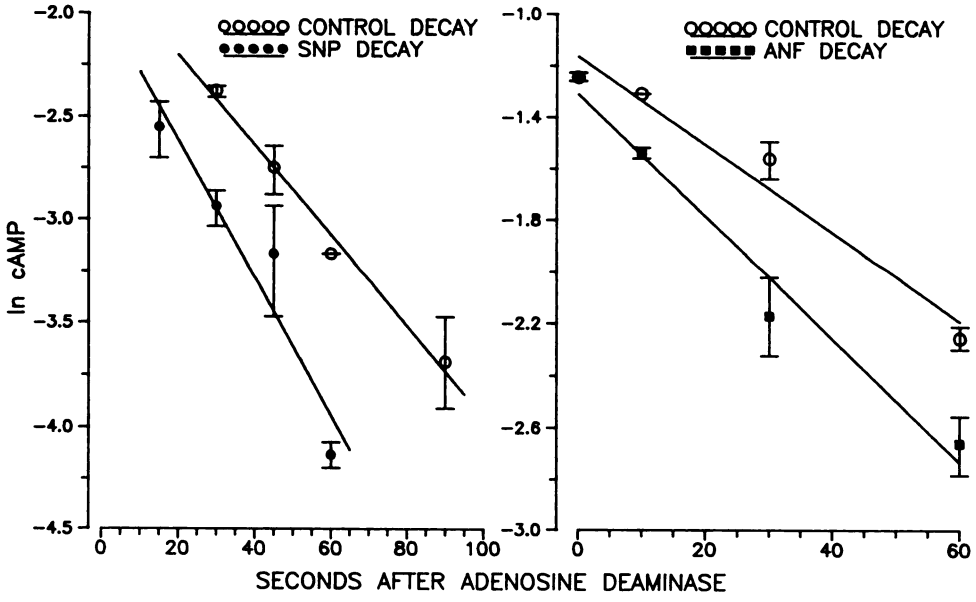

Fig. 7. SNP- and ANF-stimulated cAMP decay in PC12 cells. PC12 cells were prelabeled with [^{3}H]adenine and incubated with 50 μM adenosine for 5 min, as described in the legend to Fig. 3. Cyclic AMP accumulation was terminated by the addition of adenosine deaminase (0.6 unit), and cAMP decay was monitored for the indicated times. Percentages of cAMP conversion were transformed to ln cAMP in order to determine the rate of cAMP decay according to the method of Barber *et al.* (21). *Left*, effect of addition of 500 μM SNP with the deaminase to initiate decay; *right*, effect of addition of 1 μM ANF with the deaminase. Data for the right panel were transformed and replotted from a preliminary publication (16). The means of triplicate determinations are plotted ± standard deviation. The *solid lines* are fitted by linear regression analysis.

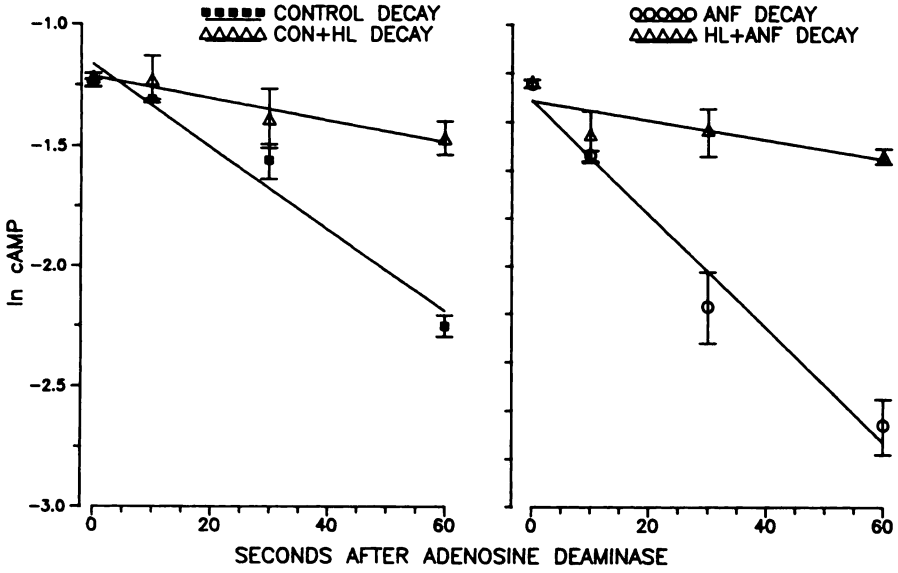

Fig. 8. HL-725 inhibition of basal and ANF-stimulated cAMP decay in PC12 cells. PC12 cells were prelabeled and incubated with 50 μM adenosine for 5 min, and their cAMP accumulation was terminated with adenosine deaminase, as described in the legend to Fig. 7. The data for both panels were transformed and replotted using values published previously (16). *Left*, effect of addition of 100 μM HL-725 with adenosine deaminase to terminate adenosine-induced cAMP accumulation; *right*, effect of addition of HL-725 with adenosine deaminase and 1 μM ANF to the cell incubation medium. Linear regression lines are shown for the means ± standard deviations of triplicate determinations from a representative experiment.

Discussion

The results of these studies demonstrate that cell-free fractions of PC12 cells show a unique profile of cyclic nucleotide PDE activity. PDE II, cGMP-activatable PDE, is the major cAMP hydrolytic activity present in PC12 cells. Crude and highly purified enzyme fractions show properties (subunit size, kinetics, and cGMP regulation) similar to those for PDE II forms purified from rabbit brain particulate fraction (12) or other tissue sources (11). The highly purified rat PC12 PDE II has a lower specific activity (approximately 8 μmol/min/mg) than the purified rabbit brain particulate enzyme (160 μmol/min/mg). The PC12 enzyme is inhibited by isoquinolines but is not inhibited by other isozyme-selective agents, e.g., CGS 9343B, indolidan, zaprinast and rolipram.

Studies of intact PC12 cell cAMP accumulation suggest that PDE II is a cGMP receptor in these cells. PC12 cells accumulate cAMP rapidly in response to adenosine, indicating a functional coupling between adenosine receptors, guanine nucleotide-binding proteins, adenylate cyclase, and cyclic nucleotide PDE activities. The isoquinoline compounds HL-725 and papaverine are the only PDE inhibitors tested that potentiate adenosine-stimulated cAMP accumulation in PC12 cells (16). The decay of cAMP in PC12 cells is also very rapid after the termination of adenosine-stimulated cAMP accumulation by the addition of exogenous adenosine deaminase; cAMP levels return to basal values within 2 min. The rate of cAMP decay is retarded in the presence of HL-725, compared with control. HL-725 also increases basal levels of cAMP, suggesting that an HL-725-sensitive enzyme, presumably PDE II, is involved in the regulation of both basal and agonist-stimulated cAMP levels in PC12 cells. Thus, inhibition of PDE II in cell-free fractions and potentiation of cAMP accumulation and inhibition of cAMP decay in the intact cell by incubation with HL-725 strongly indicate that PDE II regulates cAMP metabolism in PC12 cells.

ANF and SNP, activators of particulate and soluble guanylate cyclase activities, respectively (37, 38), increase intracellular cGMP levels and attenuate adenosine-stimulated cAMP accumulation in PC12 cells. Additional evidence in support of the stimulation of PDE II by cGMP in intact cells is the finding that HL-725 is able to block the attenuating effect of the cGMP-elevating agents. In contrast, the cGMP analogs 8-bromo-cGMP and $N^2,2'$-$O^{2'}$-dibutyryl-cGMP do not stimulate cAMP hydrolysis by PDE II or mimic the effects of ANF or SNP on attenuation of the adenosine response (16). These analogs are, however, potent activators of cGMP-dependent protein kinase (39). The observation that immunoreactive cGMP-dependent protein kinase could not be detected in PC12 cells suggests that PDE II is the major and possibly the only cGMP receptor present in these cells. The fact that ANF and SNP produce similar attenuation of adenosine-stimulated cAMP accumulation argues that these effects are not mediated by inhibition of adenylate cyclase.

An alternative explanation for the reduction of cAMP in response to ANF or SNP is a cGMP-stimulated egress of cAMP from the cells. This possibility has been investigated by performing cAMP accumulation experiments in the presence of probenecid, an organic acid transport inhibitor that inhibits cAMP egress (40, 41). We found that ANF and SNP attenuated cAMP accumulation to the same extent in the absence or presence of probenecid, suggesting that egress is not responsible for the decrease in cellular cAMP levels (data not shown).

If the attenuation of adenosine-stimulated cAMP accumulation by ANF or SNP is mediated via a cGMP-dependent activation of PDE II, then the rate of cAMP hydrolysis in the presence of these agents would be expected to be enhanced, compared with controls. The rates of cyclic AMP decay in the presence of ANF or SNP are accelerated, compared with control decay values. Thus, an interpretation of these data is that increased intracellular cGMP levels activate PDE II, resulting in a decrease in the apparent K_m of the enzyme for cAMP with no effect on maximum velocity. This change in apparent K_m is manifested as an increase in the V_{max}/K_m ratio, which, in turn, results in the increase in the rate of cAMP decay. This contention is further supported by the finding that HL-725 retards the rate of cAMP decay in the presence of ANF. The plots of cAMP decay are fitted as linear functions. However, because cGMP levels are increasing during the course of our decay analyses, more extensive study of individual decay curves may prove that these functions are nonlinear.

In conclusion, these findings provide evidence to support the primary involvement of PDE II in regulating cAMP metabolism in PC12 cells. The experiments in the intact PC12 cell show that cAMP metabolism can be modulated by agents that alter the catalytic status of PDE II. Adenosine-stimulated cAMP accumulation is potentiated by enzyme inhibition and attenuated by agents that elevate intracellular cGMP. Cyclic AMP decay is retarded by PDE II inhibitors and accelerated by agents that increase cGMP content. The utility of HL-725 in these studies is derived not from its isozyme selectivity but rather from the unique isozyme content of PC12 cells. These studies have also established the value of the PC12 cell line as a model system for future studies related to the regulation of PDE II. The potential role of this unique phosphodiesterase isozyme as a cGMP receptor site for the regulation of cAMP-mediated cellular processes is intriguing.

Acknowledgments

The authors thank Dr. T. M. Lincoln and Ms. Laurie Wear for assistance in performing the cGMP-dependent protein kinase immunoblots and Dr. C.-C. Shen for his excellent technical assistance.

References

1. Greene, L. A., and A. S. Tischler. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**:2424–2428 (1976).
2. Noronha-Blob, L., R. P. Marshall, W. J. Kinnier, and D. C. U'Prichard. Pharmacological profile of adenosine A_2 receptors in PC12 cells. *Life Sci.* **39**:1059–1067 (1986).
3. Williams, M., M. Abreu, M. F. Jarvis, and L. Noronha-Blob. Characterization of adenosine receptors in the PC12 pheochromocytoma cell line using radioligand binding: evidence for A_2 selectivity. *J. Neurochem.* **48**:498–502 (1987).
4. Rathinavelu, A., and G. E. Isom. High affinity receptors for atrial natriuretic factor in PC12 cells. *Biochem. Biophys. Res. Commun.* **156**:78–85 (1988).
5. Fiscus, R. R., B. T. Robles, S. A. Waldman, and F. Murad. Atrial natriuretic factors stimulate accumulation and efflux of cyclic GMP in C6-2B rat glioma and PC12 rat pheochromocytoma cell cultures. *J. Neurochem.* **48**:522–527 (1987).
6. Rabe, C. S., and R. McGee, Jr. Regulation of depolarization-dependent release of neurotransmitters by adenosine: cyclic AMP-dependent enhancement of release from PC12 cells. *J. Neurochem.* **41**:1623–1634 (1983).
7. Rabe, C. S., J. Schneider, and R. McGee, Jr. Enhancement of depolarization-dependent neurosecretion from PC12 cells by forskolin-induced elevation of cyclic AMP. *J. Cyclic Nucleotide Res.* **8**:371–384 (1982).
8. Drewett, J., G. Marchand, R. Ziegler, and G. Trachte. Atrial natriuretic factor inhibits norepinephrine release in an adrenergic clonal cell line (PC12). *Eur. J. Pharmacol.* **150**:175–179 (1988).
9. Drewett, J., R. Ziegler, G. Marchand, and G. Trachte. Cyclic guanosine 3′,5′monophosphate mediates the inhibitory effect of atrial natriuretic factor in adrenergic neuronal pheochromocytoma cells. *J. Pharmacol. Exp. Ther.* **250**:428–432 (1989).
10. Strada, S. J., M. W. Martin, and W. J. Thompson. General properties of multiple molecular forms of cyclic nucleotide phosphodiesterase in the nervous system. *Adv. Cyclic Nucleotide Res.* **16**:13–29 (1984).
11. Beavo, J. A. Multiple isozymes of cyclic nucleotide phosphodiesterase. *Adv. Second Messengers Phosphoprotein Res.* **22**:1–38 (1988).
12. Whalin, M. E., S. J. Strada, and W. J. Thompson. Purification and partial characterization of membrane-associated Type II (cGMP-activatable) cyclic nucleotide phosphodiesterase from rabbit brain. *Biochim. Biophys. Acta* **972**:79–94 (1988).
13. Murashima, S., T. Tanaka, S. Hockman, and V. Manganiello. Characterization of particulate cyclic nucleotide phosphodiesterases from bovine brain: purification of a distinct cGMP-stimulated isoenzyme. *Biochemistry* **29**:5285–5292 (1990).
14. Martins, T. J., M. C. Mumby, and J. A. Beavo. Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. *J. Biol. Chem.* **257**:1973–1979 (1982).
15. Beavo, J. A., J. G. Hardman, and E. W. Sutherland. Stimulation of adenosine 3′,5′-monophosphate hydrolysis by guanosine 3′,5′-monophosphate. *J. Biol. Chem.* **246**:3841–3846 (1971).
16. Whalin, M. E., S. J. Strada, J. G. Scammell, and W. J. Thompson. Regulation of cAMP metabolism in PC12 cells by type II (cGMP-activatable) cyclic nucleotide phosphodiesterase, in *Purines in Cellular Signalling: Targets for New Drugs* (K. Jacobson, J. Daly, and V. Manganiello, eds.). Springer-Verlag, New York, 323–328 (1990).
17. Epstein, P. M., S. J. Strada, K. Sarada, and W. J. Thompson. Catalytic and kinetic properties of purified high-affinity cyclic AMP phosphodiesterase from dog kidney. *Arch. Biochem. Biophys.* **218**:119–133 (1982).
18. Thompson, W. J., P. M. Epstein, and S. J. Strada. Purification and characterization of a high affinity cyclic adenosine monophosphate phosphodiesterase from dog kidney. *Biochemistry* **18**:5228–5237 (1979).
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
20. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
21. Barber, R., T. J. Goka, and R. W. Butcher. Role of high affinity cAMP phosphodiesterase activities in the response of S49 cells to agonist. *Mol. Pharmacol.* **32**:753–759 (1988).
22. Whalin, M. E., R. L. Garrett, W. J. Thompson, and S. J. Strada. Correlation of cell-free brain cyclic nucleotide phosphodiesterase activities to cyclic AMP decay in intact brain slices. *Second Messengers Phosphoproteins* **12**:311–325 (1989).
23. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
24. Brooker, G., J. F. Harper, W. L. Terasaki, and R. D. Moylan. Radioimmunoassay of cyclic AMP and cyclic GMP. *Adv. Cyclic Nucleotide Res.* **10**:1–33 (1979).
25. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
26. Munson, P., D. Rodbard, and M. L. Jaffe. CURVEFIT, IBM PC version. National Institutes of Health, Bethesda, MD (1982).
27. Yamamoto, T., V. C. Manganiello, and M. Vaughan. Purification and characterization of cGMP-stimulated cyclic nucleotide phosphodiesterase from calf liver. *J. Biol. Chem.* **258**:12526–12533 (1983).
28. Erny, R., M. W. Berezo, and R. L. Perlman. Activation of tyrosine 3-monooxygenase in pheochromocytoma cells by adenosine. *J. Biol. Chem.* **256**:1335–1339 (1981).
29. Guroff, G., G. Dickens, D. End, and C. Londos. The action of adenosine analogs on PC12 cells. *J. Neurochem.* **37**:1431–1439 (1981).
30. Norman, J. A., J. Ansell, G. A. Stone, L. P. Wennogle, and J. W. F. Wasley. CGS 9343B, a novel, potent and selective inhibitor of calmodulin activity. *Mol. Pharmacol.* **31**:535–540 (1987).
31. Kaufmann, R. F., G. V. Crow, G. G. Utterback, and D. W. Robertson. LY 195115: a potent, selective inhibitor of cyclic nucleotide phosphodiesterase located in the sarcoplasmic reticulum. *Mol. Pharmacol.* **30**:609–616 (1988).
32. Schultz, J. E., and B. H. Schmidt. Rolipram, a stereospecific inhibitor of calmodulin-independent phosphodiesterase, causes *beta*-adrenergic subsensitivity in rat cerebral cortex. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **333**:23–30 (1986).
33. Nemoz, G., A.-F. Prignet, M. Moueqqit, S. Fougier, O. Mascovschi, and H. Pacheco. Selective inhibition of one of the cyclic AMP phosphodiesterases from rat brain by the neurotropic compound rolipram. *Biochem. Pharmacol.* **34**:2997–3000 (1985).
34. Davis, C. N. Assessment of selective inhibition of rat cerebral cortical calcium-dependent and calcium-independent phosphodiesterases in crude extracts using deoxycyclic AMP and potassium ions. *Biochim. Biophys. Acta* **797**:354–362 (1984).
35. Gillespie, P. G., and J. A. Beavo. Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22948. *Mol. Pharmacol.* **36**:773–781 (1989).
36. Thomas, M. K., S. H. Francis, and J. D. Corbin. Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. *J. Biol. Chem.* **265**:14964–14970 (1990).
37. Waldman, S. A., R. M. Rapoport, and F. Murad. Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cGMP in rat tissues. *J. Biol. Chem.* **259**:14332–14337 (1984).
38. Bohme, E., G. Grossman, J. Hertz, A. Mulsch, C. Spies, and G. Schultz. Regulation of cyclic GMP formation by soluble guanylate cyclase: stimulation by NO-containing compounds. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **17**:259–266 (1984).
39. Lincoln, T. M., and J. D. Corbin. Characterization and biological role of the cGMP-dependent protein kinase. *Adv. Cyclic Nucleotide Res.* **15**:139–192 (1983).
40. Rindler, M. J., M. M. Bashor, N. Spitzer, and M. H. Saier, Jr. Regulation of adenosine 3′,5′-monophosphate efflux from animal cells. *J. Biol. Chem.* **253**:5431–5436 (1978).
41. Barber, R., and R. W. Butcher. The egress of cyclic AMP from metazoan cells. *Adv. Cyclic Nucleotide Res.* **15**:119–138 (1983).

Send reprint requests to: W. J. Thompson, Department of Pharmacology, University of South Alabama College of Medicine, Mobile, AL 36688.