Biochemical Pharmacology, Vol. 51, pp. 1739–1747, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00167-0

Rapid Regulation of a Cyclic AMP-Specific Phosphodiesterase (PDE IV) by Forskolin and Isoproterenol in LRM55 Astroglial Cells

Vergine Madelian and Eileen La Vigne*

LABORATORY OF NEUROTOXICOLOGY AND NERVOUS SYSTEM DISORDERS, WADSWORTH CENTER FOR LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY, NY 12201-0509, U.S.A.

ABSTRACT. Elevation of intracellular cyclic AMP (cAMP) levels by incubation of intact LRM55 astroglial cells with 0.1 mM forskolin or 0.1 μM isoproterenol (IPR) caused a rapid increase in soluble cAMP phosphodiesterase (PDE) activity. Activation did not require *de novo* protein synthesis and reached a maximum of ≥100% increase over basal PDE activity after 15 min of treatment. The increase in activity was recovered in a single peak (peak 3) following DEAE chromatography; the other two peaks separated by this procedure showed no change. Peak 3 had all the characteristics of PDE IV: it was sensitive to rolipram, was insensitive to CI-930 and cyclic GMP (cGMP), had a high affinity for cAMP ($K_m \simeq 4$ μM), and had a very low affinity for cGMP ($K_m > 100$ μM). Forskolin treatment resulted in an increase of the V_{max} of peak 3 without affecting its K_m. *In vitro* treatment of peak 3 with the catalytic subunit of protein kinase A increased activity, whereas treatment with alkaline phosphatase decreased activity. The rapid activation of this specific PDE in response to forskolin and IPR represents a novel regulation of PDE IV by a mechanism that seems to involve its phosphorylation by a cAMP-dependent protein kinase. BIOCHEM PHARMACOL 51;12:1739–1747, 1996.

KEY WORDS. phosphodiesterase; cAMP; glia; activation; phosphorylation; protein kinase A

Cyclic nucleotide PDEs† are a diverse group of enzymes that degrade cAMP or cGMP to 5′-AMP or 5′-GMP, respectively. Several PDE isozymes are regulated by hormonal stimuli [1]. For example, PDE III, the cGMP-inhibited PDE, is activated by prostaglandins in fibroblasts [2], by forskolin in human platelets [3, 4], by insulin and IPR in adipocytes [5, 6], and by insulin and glucagon in hepatocytes [7, 8]. Similarly, activity of PDE IV, the rolipram-sensitive PDE, is elevated in the C_6 glial cells [9, 10], in Sertoli cells [9], and in the U937 human monocyte cell line [11] after treatment with agents that elevate intracellular cAMP levels. But unlike activation of PDE III, which is short-term and mediated through the action of protein kinases [12, 13], regulation of PDE IV is long-term and involves synthesis of new enzyme [9, 11, 14].

The present study is the first report of a rapid, short-term regulation of PDE IV, one that is clearly independent of new protein synthesis. Our previous studies with intact cells have shown that cAMP regulation in astroglia occurs mainly through changes in PDE activity [15]. We have since established that 80–90% of the total PDE activity in these cells is found in the soluble (non-particulate) fraction of cell homogenates, and that approximately 75% of this soluble activity is accounted for by a rolipram-sensitive isozyme, PDE IV, the rest representing a Ca^{2+}/CaM-sensitive isozyme, and a third, as yet unidentified, PDE (unpublished observations). The present study demonstrated that in LRM55 astroglial cells two separate routes of elevating intracellular cAMP levels bring about a rapid activation of PDE IV without affecting other PDE isozymes.

MATERIALS AND METHODS

Chemicals

IPR, cycloheximide, cAMP, cGMP, the catalytic subunit of cAMP-dependent PKA, PKI, and bovine liver (type IX) AP were purchased from the Sigma Chemical Co., St. Louis, MO; forskolin was purchased from Calbiochem, La Jolla, CA; all tritiated compounds were purchased from Amersham, Arlington Heights, IL. Rolipram was a gift from Berlex Laboratories, Cedar Knolls, NJ; CI-930 was a gift from Parke–Davis, Warner–Lambert Co., Ann Arbor, MI. Ro 20-1724 was a gift from Hoffmann-La Roche, Nutley, NJ.

* Corresponding author. Tel. (518) 486-5787; FAX (518) 474-8590; E-mail: madelian@wadsworth.org

† *Abbreviations:* PDE, phosphodiesterase; cAMP, cyclic AMP; cGMP, cyclic GMP; PKA, protein kinase A; PKI, PKA-specific inhibitor peptide; HHS, HEPES-buffered Hanks' saline; AP, alkaline phosphatase; IPR, isoproterenol; CaM, calmodulin; MES, 2-[*N*-morpholino]ethanesulfonic acid; and TLCK, Nα-*p*-tosyl-L-lysine chloromethyl ketone.

Received 9 August 1995; accepted 16 January 1996.

Cell Preparation

LRM55 cells originally derived from a rat spinal cord tumor [16] were grown in 100-mm plastic culture dishes as previously described [17].

Enzyme Preparation

Cells grown to confluency were washed with HHS, scraped off the plates, homogenized in assay buffer (40 mM Tris–Cl, pH 8.0, 4 mM 2-mercaptoethanol), and centrifuged at 100,000 *g* for 45 min, 4°. The supernatant fraction either was used directly to measure soluble PDE or was loaded onto a DEAE column for the separation of PDE isozymes.

DEAE Chromatography

Three milliliters (containing 1.5 to 2 mg protein) as a concentrated soluble preparation was loaded on a 7.5 mm × 7.5 cm column (Waters Protein Pak DEAE-5PW) fitted in a Pharmacia FPLC system. The column was washed with 5 mL of low salt buffer (5 M MES, pH 6.5, 2 mM mercaptoethanol, 15 mM sodium acetate, 20 μM TLCK as a protease inhibitor, and 1 mM EGTA), eluted with 20 mL of a linear sodium acetate gradient (15 mM to 1.2 M), and washed with 5 mL of high-salt buffer (containing 1.2 M sodium acetate). Half-minute (0.5 mL) fractions were collected on ice, and 100- or 200-μL aliquots were assayed.

PDE Assay Procedure

Assays were started by adding 100–200 μL enzyme preparations to a reaction mix (total volume of 400 μL) containing Tris–Cl (40 mM pH 8.0), 2-mercaptoethanol (4 μM), $MgCl_2$ (4 mM), [^{3}H]cAMP (400,000 cpm), and unlabeled cAMP (1 μM, or as indicated) and were carried out at 30° in a shaking water bath for 10 min (or as indicated). The reaction was stopped by immersing tubes in a boiling water bath for 1 min. Tube contents were centrifuged (10,000 *g*, 5 min), and 50–100 μL of the clear supernatant liquid was injected onto an HPLC column (Waters C18 μBondapak or Rainin Microsorb-MVC18). AMP was separated from cAMP using a mobile phase of 0.1 M $(NH_4)_2HPO_4$, pH 6.0, and 10% methanol, pumped at 1 mL/min. Fractions (0.5 mL) were collected throughout the run and counted for radioactivity by liquid scintillation counting, or the eluate was passed through a flow-through scintillation counter (Radiomatic A-200). PDE activity was calculated by converting radioactivity in the AMP peak to pmol AMP/(min · tube) or pmol AMP/(min · mg protein). When assaying for cGMP degradation, tritiated and unlabeled cGMP were substituted for cAMP.

cAMP Contents

cAMP contents were measured as previously described [15]. Briefly, the intracellular ATP pool of intact cells was labeled by incubating cells with [2-^{3}H]adenine (10 μCi/mL). cAMP production was stimulated with the addition of agonist, and the reaction was stopped with 0.3 M NaOH. Proteins and non-cyclic nucleotides were precipitated with the addition of 0.75 M $ZnSO_4$. cAMP in the supernatant liquid was separated from [^{3}H]adenine and other ^{3}H-labeled derivatives by HPLC on a Novapak C18 Radial-PAK cartridge (Waters Associates, Milford, MA) using a mobile phase of [0.1 M $(NH_4)_2HPO_4$, 1% methanol, pH 3.0]. The elution of cAMP from the column was monitored at 254 nm, and the radioactivity associated with it was measured by liquid scintillation counting.

In Vitro *Treatment with PKA*

In vitro activation was carried out in four 1.5-mL centrifuge tubes (400 μL total volume each), using the catalytic subunit of PKA [12]. Aliquots (200 μL) of peak 3 in 40 mM Tris–Cl, pH 7.4, 0.5 mM dithiothreitol, 50 μM sodium ortho-vanadate, 10 mM $MgCl_2$, 0.2 mM ATP (in tubes 1, 2, and 4), and 500 U PKI (in tube 4 only) were preincubated in a 30° shaking water bath for 2 min. Reactions were started by adding 40 U of PKA to tubes 2, 3, and 4, and incubations were continued for 10 min. Triplicate 100-μL aliquots were removed from each tube and assayed for PDE activity in the presence of 50 μM sodium ortho-vanadate for 5 min. Thus, although samples were treated with PKA for 10 min, PKA was also active during the 5-min PDE assay. Reactions were stopped, and PDE activity was determined as described earlier.

In Vitro *Treatment with AP*

Duplicate 100-μL aliquots of a peak 3 preparation were added to 100 μL of 40 mM Tris–Cl, pH 9.9, with or without 0.05 units AP, or to 100 μL Tris–Cl, pH 8.0. Incubations were carried out in a shaking water bath at 30° for 3 min. PDE assay mixture was added directly to these tubes, and the incubation was continued for an additional 5 min to determine PDE activity. Again, although samples were treated with AP for only 3 min, the enzyme was still active, although diluted, during the 5-min PDE assay.

Protein Contents

Protein was determined using the Bradford dye-binding method with bovine serum albumin as standard [18].

RESULTS

Time Course of Activation of Soluble PDE

When LRM55 astroglial cells are incubated with IPR, cAMP levels rise rapidly and then fall slowly [17]. Our previous studies have indicated that the decline in cAMP levels is due primarily to increased PDE activity, and suggested that this is brought about by elevated cAMP levels [15]. To determine whether elevation of cAMP levels does indeed cause an increase in PDE activity, we activated ad-

enylate cyclase by treating intact cells with 0.1 μM IPR (β-adrenergic receptor-mediated activation) or with 0.1 mM forskolin (receptor-independent activation). These concentrations were chosen because they were shown previously to elicit near-maximal cAMP responses in these cells [15]. Time courses of cAMP accumulation (Fig. 1, dotted lines) showed that both forskolin (Fig. 1A) and IPR (Fig. 1B) treatments caused intracellular cAMP levels to rise rapidly from a baseline of 0.16 pmol/tube to a maximum of 10.4 pmol/tube (forskolin) or 20.2 pmol/tube (IPR). The cAMP response to IPR was both faster (5 min to reach maximum) and larger (125% increase over baseline) than that to forskolin (10 min, 65% increase). With both treatments, PDE activity also increased in a time-dependent fashion from a baseline of 12 pmol/(min · tube) to 23 pmol/(min · tube) with forskolin (Fig. 1A, solid line) and 34 pmol/(min · tube) with IPR (Fig. 1B, solid line). The increase in PDE activity followed, with a slight time-lag, that of intracellular cAMP levels, it was also faster, and reached a higher maximal value in IPR- compared with forskolin-treated cells. With both treatments, decline in cAMP levels was not detectable until PDE activity reached a critical level (approximately 100% increase over baseline); once above this level, the higher PDE activity reached in IPR-treated cells correlated with the more rapid drop of intracellular cAMP levels in these cells. Upon prolonged (30 min) incubation, as cAMP levels declined, so did PDE activity. These results were consistent with our hypothesis that an increase in PDE activity was brought about by elevated cAMP levels, and that once elevated, PDE activity caused intracellular cAMP levels to drop.

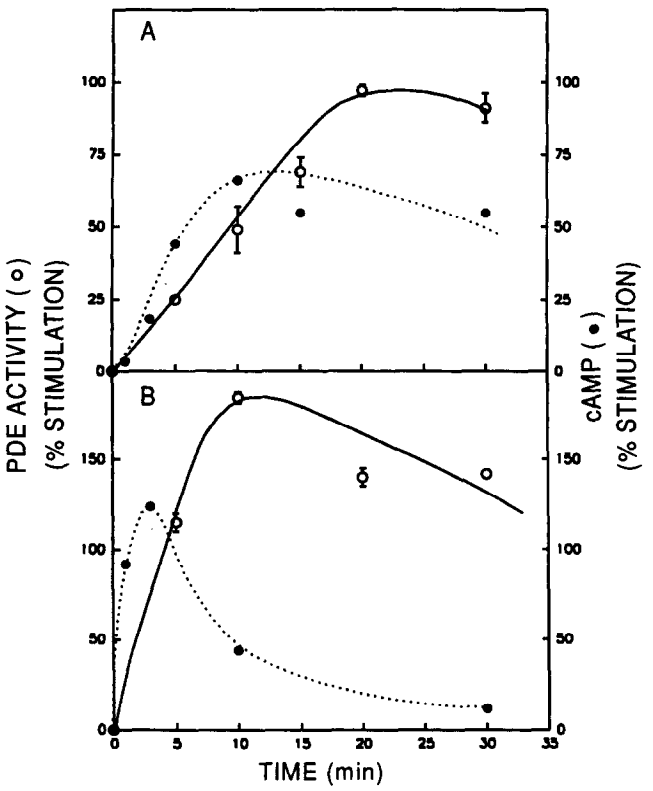

FIG. 1. Time course of PDE activation. LRM55 cells grown to confluency in 100-mm culture dishes were washed with HHS and incubated at 37° with 0.1 mM forskolin (A) or 0.1 μM IPR (B) for the indicated periods. The drugs were then aspirated, and the cells were washed three times with 1 mL buffer each, scraped off the dishes, and homogenized in assay buffer (1 mL/dish). The homogenate was centrifuged at 100,000 *g* for 45 min at 4°, and duplicate 200-μL aliquots of the supernatant liquid were assayed for PDE activity using 1 μM cAMP as substrate. For cAMP measurements, sister plates preincubated with [^{3}H]adenine were washed three times with 1 mL HHS each, and exposed to IPR or forskolin for the indicated periods. Cells were processed as described in Materials and Methods, nucleotides were separated by HPLC, and cAMP contents were determined by scintillation counting. Both cAMP contents and PDE activity are expressed as percent stimulation over basal levels, which were 0.16 pmol cAMP/tube and 12 pmol PDE/(min · tube). Each point represents the mean ± SD of three experiments, each carried out in duplicate.

Protein Synthesis and PDE Activation by Forskolin

To determine whether this activation required synthesis of new enzyme, cells were treated with forskolin in the presence and absence of 50 μM cycloheximide, a concentration previously determined to inhibit protein synthesis (measured by [^{3}H]leucine incorporation into perchloric acid-precipitable material (Fig. 2 inset). The presence of cycloheximide had no effect on the forskolin-induced activation of PDE, since cells treated with forskolin, with or without cycloheximide, showed the same degree of activation, i.e. a near doubling over untreated cells (Fig. 2). Similar results were obtained with IPR (data not shown).

Which PDE Is Activated?

Soluble PDE activity in LRM55 cells is due to several isozymes. As a first step in determining which isozymes were activated, they were separated by ion exchange chromatography using an FPLC system (see Materials and Methods) into three distinct activity peaks that we have called peaks 1, 2, and 3, based on their order of elution from the column (Fig. 3). Peaks 1 and 2 hydrolyzed both cAMP and cGMP, whereas peak 3 was cAMP-specific (Fig. 3A). Peak 1 was inhibited by the addition of EGTA and greatly stimulated by Ca^{2+}/CaM (data not shown). Peak 2 is not fully characterized. Peak 3 was insensitive to Ca^{2+}/CaM and cGMP (not shown). It was not inhibited by CI-930, but 0.25 μM rolipram (a concentration that corresponds to the IC_{50} of most type IV PDEs) inhibited it at approximately 50% (Fig. 3B). Based on its substrate preference and its inhibitor pharmacology, we concluded that peak 3 was a type IV PDE isozyme. To determine (a) whether forskolin-induced activation of PDE was retained after DEAE chromatography, and (b) which peak showed an increase in activity, soluble preparations from forskolin-treated and untreated cells were chromatographed on a DEAE column, and the eluted fractions were assayed. Activities in peaks 1 and 2 were similar in control and forskolin-treated cells, but peak 3 activity

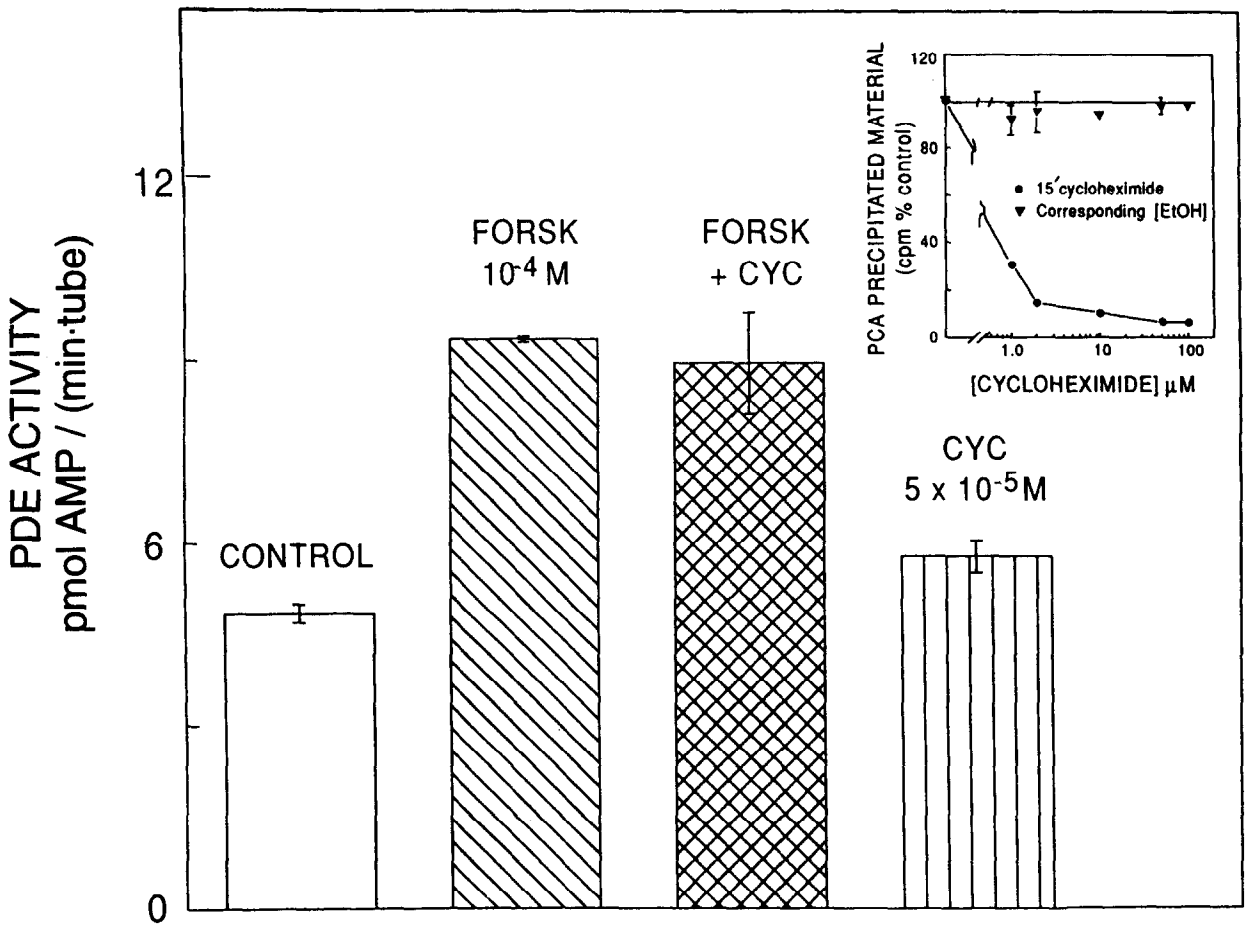

FIG. 2. Effect of cycloheximide on stimulation of PDE activity by forskolin. Confluent cultures of cells were incubated for 15 min with or without 0.1 mM forskolin in the presence or absence of 50 μM cycloheximide. The medium was aspirated, and cells were washed, scraped, and homogenized. The homogenate was centrifuged, and duplicate 200-μL aliquots of each supernatant liquid were assayed using 1 μM cAMP as substrate. Each point represents the mean ± SD of 3 experiments. Protein 20–23 μg/200 μL. Inset. Effect of cycloheximide concentration on [^{3}H]leucine incorporation into perchloric acid (PCA)-precipitable material. Cells grown in 24-well trays were washed and incubated for 15 min with 1.25 μCi [^{3}H]leucine and various concentrations of cycloheximide (●) or the corresponding concentrations of ethanol (▼), used to dissolve the cycloheximide. The medium was aspirated, cells were washed three times with 1 mL HHS each, and 250 μL of ice-cold 0.4 N PCA was added to each well. After an overnight incubation at 4°, contents of each well were dispersed by sonication; wells were washed with ice-cold PCA, which was added to the sonicate, and spun at 10,000 g for 5 min. The PCA precipitate was dissolved in 250 μL of 0.4 N NaOH, and the radioactivity was determined by scintillation counting. Two wells were used for each condition. Each point represents the mean ± SD of three experiments. Cells incubated with [^{3}H]leucine in the absence of cycloheximide were used as controls. Both these and the ethanol controls incorporated 260,000–263,000 cpm/well into PCA-precipitable material. Protein = 130 ± 10 μg/well.

was significantly higher in forskolin-treated cells (Fig. 4). Treatment of cells with IPR also resulted in an increase in peak 3 activity (Fig. 4, inset), without affecting peaks 1 and 2. Because we had characterized peak 3 previously as a PDE IV isozyme, this increase in peak 3 activity strongly suggested that the activated PDE was this particular isoform. However, another low-K_m isozyme, PDE III, often co-elutes with PDE IV during DEAE chromatography, and although we have detected no PDE III activity in these cells, we could not rule out the possibility that it could be present in an inactive form in control cells, and be activated in response to forskolin or IPR treatment. To ascertain that the forskolin-treated peak 3 was PDE IV, and contained no PDE III, we first determined that the forskolin-treated peak 3 was not inhibited by CI-930, even at concentrations as high as 1 μM (data not shown). We then established that the sensitivity of peak 3 to rolipram did not change following treatment (IC_{50} = 0.5 vs. 0.6 μM, see Fig. 5, A and B, filled circles). Next, we showed that for both control and forskolin-treated peaks 3 the rolipram inhibition curves did not shift to the left in the presence of 1 μM CI-930 (Fig. 5, A and B), as would be expected if even small amounts of PDE III activity were present [19]. Taken together, these pharmacologic data indicate that the forskolin-treated peak 3, much like the native one, is comprised of PDE IV, and that neither contains PDE III.

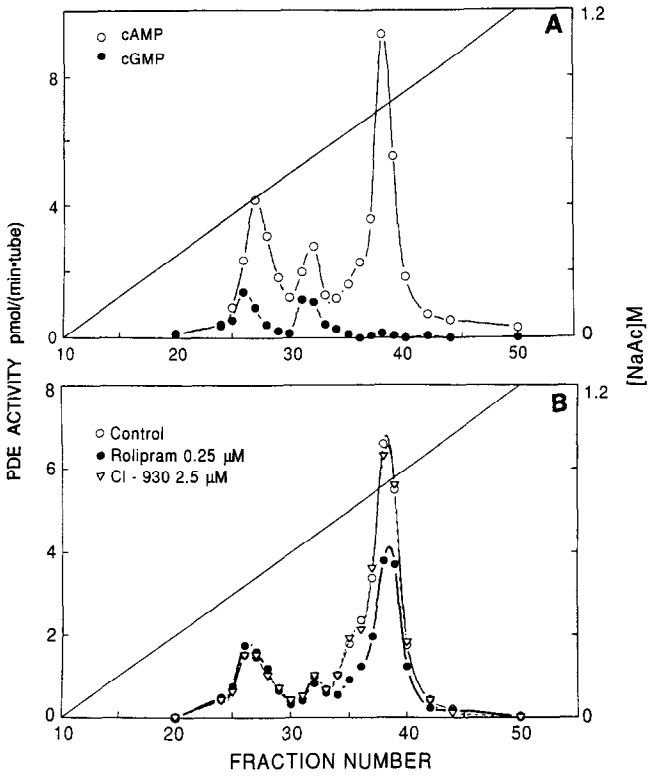

FIG. 3. Separation of the three PDE activity peaks by DEAE chromatography. Soluble PDE was prepared using cells from 10 culture plates homogenized in 5 mL of assay buffer. Three milliliters of the supernate obtained after centrifugation, containing 2–3 mg protein (2.25 mg for A, and 2.16 mg for B below) were loaded onto a DEAE column, washed with low salt buffer, and eluted with a linear gradient of sodium acetate at the rate of 1.0 mL/min. (A) Substrate specificity. Aliquots (200 μL) of each 0.5-mL fraction were assayed with 1 μM cAMP (○) or 0.5 μM cGMP (●) for 10 min. This chromatogram is one of three experiments, all showing similar patterns. Recoveries: 72% protein, 65% activity. (B) Sensitivity to inhibitors. Aliquots (100 μL) of each fraction were assayed for 20 min in the absence (control) and presence of 2.5 μM CI-930 or of 0.25 μM rolipram. PDE activity is expressed as pmol/(min · tube), i.e. pmol of cAMP or cGMP hydrolyzed per min by a 200-μL aliquot of the DEAE fraction. This chromatogram represents one of at least a dozen experiments, all showing the same pattern. Recoveries: 69% protein, 61% activity.

Kinetic Characteristics of Activated PDE

To characterize the changes in kinetic properties of cAMP hydrolysis resulting from forskolin treatment, peak 3 fractions from treated and untreated cells were pooled and assayed with several concentrations (ranging from 0.1 to 100 μM) of cAMP as substrate. In four separate experiments, forskolin-treated samples showed a significantly higher V_{max}, but no change in K_m. Data pooled from all four experiments are shown in Table 1.

Phosphorylation State and PDE Activity

To determine whether activation of peak 3 PDE was due to cAMP-dependent protein kinase (PKA) activity, a peak 3 preparation was incubated, *in vitro*, in the presence or absence of the catalytic subunit of PKA, ATP, and PKI, as described in Materials and Methods. Tube 1 contained ATP but no exogenously added PKA, tube 2 contained PKA and ATP, tube 3 contained PKA but lacked ATP, and tube 4 contained PKA, ATP, and a 10-fold excess of PKI (Fig. 6A). Following a 10-min treatment, PDE activity in tubes containing added PKA was 123 pmol/(min · tube), compared to 92 pmol/(min · tube) in control tubes containing no added PKA. Both ATP and active PKA were required for this increase (33%), since omission of either during incubation, or inhibition of PKA by PKI, abolished this activation (Fig. 6A). To assess further the relationship between phosphorylation state and PDE activity, peak 3 was treated, *in vitro*, with AP for 5 min (see Materials and Methods). PDE activity in the AP-treated samples was 28 pmol/(min · tube), compared to 76 pmol/(min · tube) in controls containing pH 9.9 buffer instead of AP, or to 84 pmol/(min · tube) in control tubes incubated with pH 8.0 PDE assay buffer (Fig. 6B). This >60% drop in PDE activity following AP treatment indicated that a certain level of phosphorylation was required even for basal levels of peak 3 activity. We should note that treatment with AP would sometimes dephosphorylate AMP to adenosine (unpublished observations), and thus yield erroneous results. We therefore took several precautions in analyzing AP-treated samples. First, HPLC eluates were scrutinized carefully during PDE assays to detect any radioactivity eluting with a retention time similar to that of adenosine; second, at the end of the PDE assay, but prior to separation and analysis by HPLC, an aliquot was further treated with adenosine deaminase: the absence of radioactive inosine (detectable by HPLC/scintillation counting) indicated the absence of adenosine in the original reaction products; and last, a second aliquot was treated with 5′-nucleotidase: the complete disappearance of radioactive AMP and the appearance of radioactive adenosine indicated that the original product was indeed AMP (data not shown).

DISCUSSION

Our previous studies have shown that continuous stimulation of LRM55 astroglial cells with β-adrenergic receptor agonists produces a rapid increase followed by a slow decline of intracellular cAMP levels [17]. The presence of PDE inhibitors during treatment abolished this decline in cAMP contents, suggesting that the apparent "desensitization" observed in these cells might reflect an increase in the degradation of cAMP rather than a decline in its synthesis [15]. Since PDEs are the only enzymes that can hydrolyze cAMP, we undertook the present study to determine whether PDE regulation was responsible for cAMP regulation in these cells, i.e. whether PDE activity increased in response to elevated cAMP levels and, if so, which PDE isozyme(s) was affected.

Activation of adenylate cyclase with forskolin or through

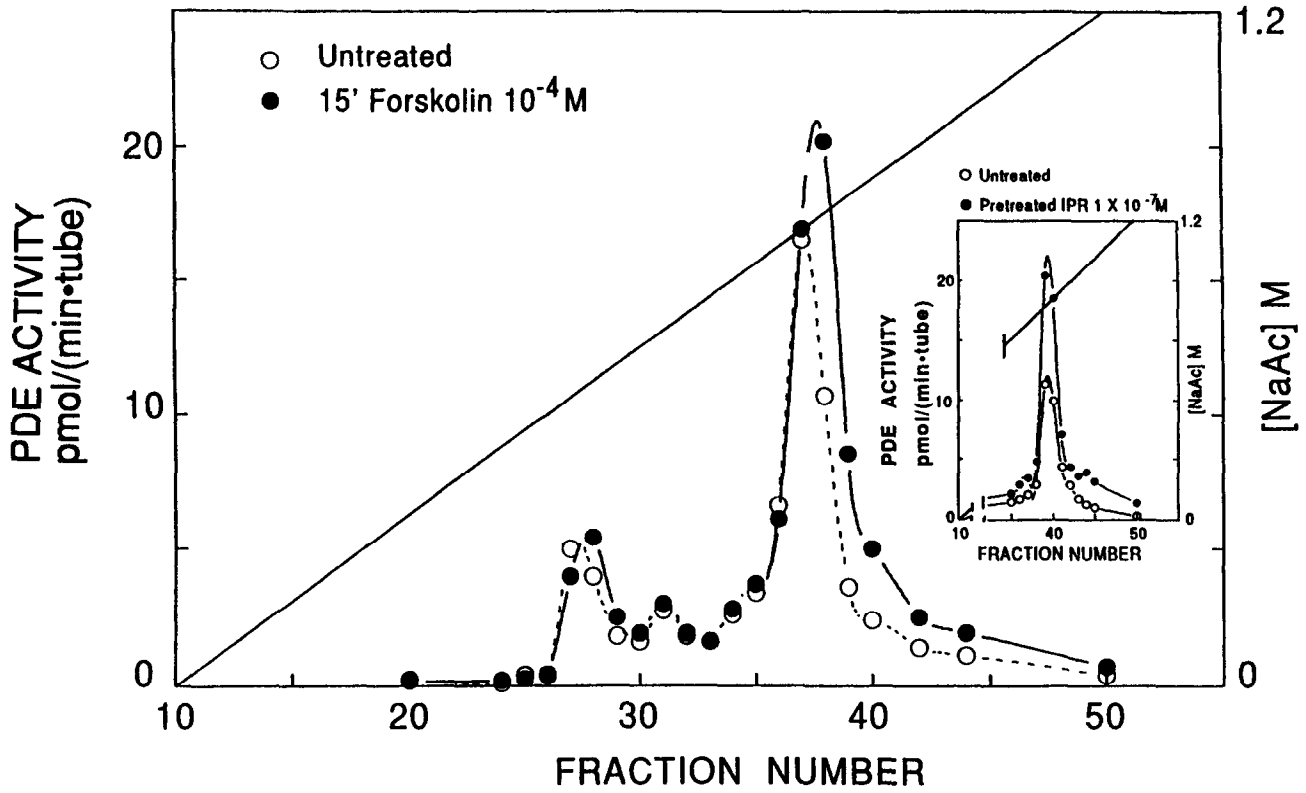

FIG. 4. Activation of peak 3 PDE after forskolin treatment of cells. Ten dishes of confluent cells were incubated for 15 min with 0.1 mM forskolin or with HHS. Incubation medium was aspirated, and cells were rinsed, rapidly scraped off the plates, homogenized (0.5 mL/plate), and centrifuged. Three milliliters (1.40 mg protein) of each supernatant liquid was loaded onto a DEAE column, and eluted with a sodium acetate gradient. Half-minute (0.5 mL) fractions were collected, and 200-μL samples were assayed for PDE activity, using 1 μM cAMP as substrate. Recoveries: control: 70% protein, 60% activity; treated: 71% protein, 59% activity. Inset: cells were treated with 10 μM IPR for 15 min prior to homogenization and chromatography. Recoveries: control: 75% protein, 64% activity; treated: 74% protein, 64% activity. Each chromatogram represents one of at least three experiments.

β-adrenergic receptor stimulation with IPR resulted in a 65–125% increase in intracellular cAMP levels (Fig. 1, A and B, dotted lines), and a 100–200% increase in soluble PDE activities (Fig. 1, A and B, solid lines). Similar to the cAMP response, the PDE response elicited by IPR was faster and stronger than that elicited with forskolin. This and the fact that PDE activation lagged slightly behind the cAMP response during the "activation" phase were consistent with the hypothesis that elevated cAMP levels resulted in increased PDE activity. We have shown previously [15] that in LRM55 cells adenylate cyclase remains very active during continuous stimulation with IPR and forskolin. Thus, at any one time, intracellular cAMP levels reflect the equilibrium between the activity of adenylate cyclase and that of phosphodiesterase. During the early phase of the time course (Fig. 1), cAMP levels rise due to the activation of adenylate cyclase by IPR or forskolin. This, in turn, causes an increase of PDE activity, which attenuates the increase in cAMP levels. Both with IPR and forskolin, an increase in PDE activity of approximately 100% seems to be required before hydrolysis of cAMP is substantial enough to overtake synthesis, and cause a net decline of intracellular cAMP levels. The sharper drop of cAMP levels in IPR- versus forskolin-treated cells reflects the higher level of PDE activity in the former.

Following treatment with forskolin or IPR, the only PDE that showed increased activity was the one that eluted as peak 3 during DEAE chromatography (Fig. 4). In both control and forskolin-treated cells, this peak displayed the characteristics of PDE IV: it showed a high affinity for cAMP ($K_m \simeq 4$ μM), was inhibited by rolipram ($IC_{50} < 1$ μM), but was not inhibited by CI-930 (Figs. 3B, 5A and 5B).

The increased activity recovered after DEAE chromatography (Fig. 4) was considerably lower than that observed in crude homogenates (approximately 50 vs 100% with forskolin treatment, and 100 vs 200% with IPR treatment). This discrepancy could indicate either that the "activated" PDE IV was more labile than its basal counterpart, and/or that the activation observed in crude preparations reflected more than that of peak 3. Overall recoveries from DEAE columns varied from run to run (usually around 60%), but they were not significantly different between control and treated cells. It is therefore difficult, based on the present results, to distinguish between these possibilities. Regardless of this uncertainty, however, the recovery of an elevated peak 3 activity from treated cells indicated that regulation

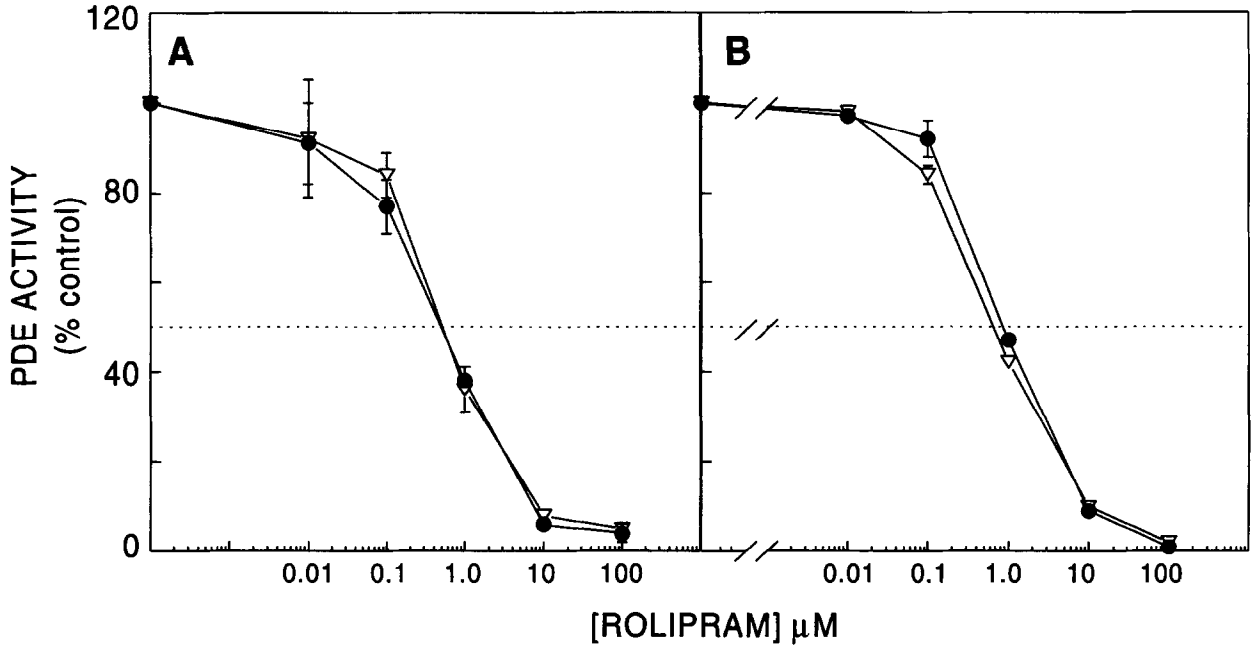

FIG. 5. Effect of CI-930 on the rolipram inhibition curves of peak 3 from control and forskolin-treated cells. Soluble preparations from control (A) and forskolin-treated (B) cells were loaded onto, and eluted from, a DEAE column. Peak 3 fractions were pooled and assayed with various concentrations of rolipram in the presence (▽) and absence (●) of 1 μM CI-930. Protein contents for pooled peak 3 samples were 38 and 45 μg/mL, and PDE activities of uninhibited samples were 5.0 and 6.8 pmol/(min · tube) for control and treated cells, respectively. Each point represents the mean ± SD of three experiments, each carried out in duplicate.

of PDE IV was not due to readily reversible changes, but that it reflected either the increase in the amounts of PDE IV through protein synthesis, or the activation of the existing enzyme through covalent modifications. Kinetic analysis of control and forskolin-treated peaks 3 (Table 1) did not differentiate between these two possibilities, since the significant kinetic effect of activation was a 25% increase in V_{max} [4.6 vs 3.7 nmol/(min · mg)]. No change in K_m (4.0 vs 4.2 μM) was observed. Similar changes in V_{max} with no change in K_m have been reported both in cases of rapid activation [20, 21] and phosphorylation [12], as well as transcriptional regulation of PDEs [11]. However, both the rapid time course (Fig. 1) and the failure of cycloheximide to interfere with this regulation (Fig. 2) argue strongly against synthesis of new enzyme, and point to a post-translational modification of existing PDE IV as the likely mechanism of activation in this instance. This is further supported by our *in vitro* results, in which a 10-min treatment of peak 3 with the catalytic subunit of PKA resulted in a 35% increase in activity (Fig. 6A). In repeated experiments, the PKA-induced activation of peak 3 varied between 25 and 35%. Although relatively modest compared with the activation observed in crude homogenates after treatment of cells with forskolin or IPR (Figs. 1 and 2), this level of activation was similar to that recovered in peak 3 following DEAE chromatography (Fig. 4), indicating that phosphorylation of peak 3 by PKA is the most likely mechanism of forskolin- and IPR-induced activation of this peak. The loss of activity of native peak 3 after treatment with AP (Fig. 6B) further suggests that phosphorylated moieties are important even for basal levels of peak 3 activity.

TABLE 1. Effect of forskolin treatment on V_{max} and K_m of peak 3

Treatment	V_{max} [nmol/(min · mg)]	*P*	K_m (μM)	*P*
Control	3.7 ± 0.6		4.0 ± 0.3	
Forskolin	4.6 ± 0.8	0.01	4.2 ± 0.2	>0.5

Ten dishes of confluent cells were incubated for 15 min with 0.1 mM forskolin or with HHS. Soluble preparations were made from each set of cells; 3 mL (2.5 to 2.7 mg protein) of each set was loaded onto a DEAE column and eluted with a sodium acetate gradient. Fractions corresponding to peak 3 were pooled from each run (145–150 μg protein/mL), and were assayed with a range of cAMP concentrations (0.1 to 100 μM) as substrate. V_{max} and K_m were obtained by regression analysis. Means ± SD and *P* values were determined by a paired *t*-test using data from 4 sets of experiments.

Hormonal regulation of specific PDE isozymes occurs in a number of tissues, and in many instances it is accomplished through phosphorylation. PDE III, for example, is activated in hepatocytes [22], platelets [12, 23], and adipocytes [13, 24–26] when intact cells are subjected to treatments that activate PKA or insulin-dependent kinase [25, 26]. The direct role of PKA and phosphorylation in the activation of PDE III was clearly established in platelets [12, 23] and adipocytes [13], when incubation of broken cell preparations with the catalytic subunit of PKA resulted in both the phosphorylation and activation of this PDE, and

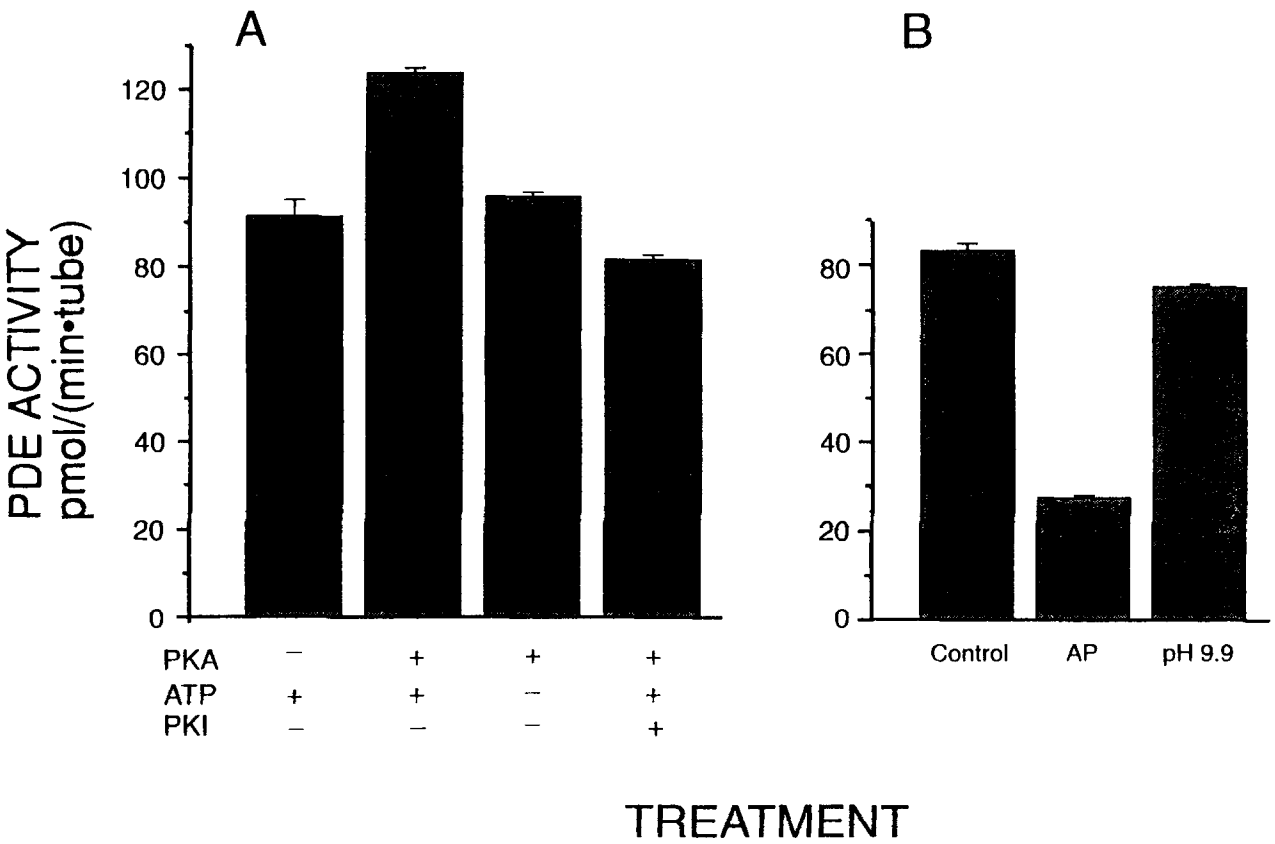

FIG. 6. Effects of *in vitro* PKA and AP treatment on peak 3 PDE activity. (A) Activation with PKA. Four tubes containing a peak 3 preparation and 40 mM Tris–Cl, pH 7.4, 0.5 mM dithiothreitol, 50 μM sodium ortho-vanadate, 10 mM $MgCl_2$, 0.2 mM ATP (in tubes 1, 2, and 4), and 500 units PKI (in tube 4 only) were preincubated for 2 min at 30° (400 μL total volume). Reactions were started by adding 40 units of the catalytic subunit of PKA to tubes 2, 3, and 4, and incubations were continued for 10 min. Triplicate 100-μL aliquots were removed from each tube and assayed for PDE activity in the presence of 50 μM sodium ortho-vanadate for 5 min. Each bar represents the mean ± SD of the triplicate samples. This graph is representative of five experiments carried out with different peak 3 preparations, all yielding similar results. (B) Inactivation with AP. Duplicate 100-μL aliquots of a peak 3 preparation were incubated for 3 min at 30° in 40 mM Tris–Cl, pH 8.0 (control), or pH 9.9, or with 0.045 units alkaline phosphatase/40 mM Tris–Cl, pH 9.9. PDE assay mixture was added directly to these tubes, and incubations were continued for an additional 5 min. PDE activity was determined as described earlier. Each bar represents the mean ± range of the duplicate samples. This graph is a representative of at least three experiments carried out with different peak 3 preparations. All yielded similar results.

when both events were inhibited by PKA-specific inhibitors. PDE I, the Ca^2/CaM-sensitive isozyme, is also regulated by phosphorylation, but here phosphorylation has the opposite effect. Bovine brain and heart contain two distinct subunits of PDE I: one of these can be phosphorylated by PKA and the other by CaM kinase [27–29]. Both phosphorylations result in decreased activity, and dephosphorylation with calcineurin restores original activity in both instances [27–29].

PDE IV is also hormonally regulated: in Sertoli cells [9, 30], U937 human monocytic cells [11], and the C6 rat glioma cells [10, 31], PDE IV activity increases following treatments that elevate intracellular cAMP levels. Unlike PDE I and PDE III, however, these regulations of PDE IV were reportedly slow, requiring 3–4 hr for the doubling of activity, and depended on protein synthesis [9, 11]. In Sertoli cells [14], where four PDE IV-specific mRNA species were identified, only one increased during treatment. Similar results indicating changes in mRNA levels corresponding to increased PDE IV activity also have been obtained with U397 cells [11], clearly establishing that PDE IV is regulated at the level of transcription. We do not know whether PDE IV in LRM55 cells is subject to long-term regulation, but our data show that in intact cells it is under rapid regulation by elevated cAMP levels, and that *in vitro* it can be activated with PKA and inactivated with AP.

In summary, it is clear that elevation of intracellular cAMP levels in LRM55 astroglial cells caused a rapid activation of PDE IV. This regulation was not due to either the indirect action of endogenous regulators on PDE, or the synthesis of new enzyme. It appears, instead, to be due to activation through a covalent and persistent modification of the enzyme itself, most likely phosphorylation by PKA.

Note Added During Revision

Since the original submisssion of this manuscript, the phosphorylation and activation of a PDE IV in thyroid FRTL-5

cells by cAMP-dependent protein kinase has been reported by Sette *et al.* (*J Biol Chem* **269:** 9245–9252, 1994).

This work was supported, in part, by NIMH RO3 47965.

References

1. Appleman MM and Terasaki WL, Regulation of cyclic nucleotide phosphodiesterase. *Adv Cyclic Nucleotide Res* **5:** 153–162, 1975.
2. Manganiello VC and Vaughan M, Prostaglandin E_1 effects on adenosine 3′:5′-cyclic monophosphate concentration and phosphodiesterase activity in fibroblasts. *Proc Natl Acad Sci USA* **69:** 269–273, 1972.
3. Grant PG, Mannarino AF and Colman RW, cAMP-mediated phosphorylation of the low-K_m cAMP phosphodiesterase markedly stimulates its catalytic activity. *Proc Natl Acad Sci USA* **85:** 9071–9075, 1988.
4. Grant PG, DeCamp DL, Bailey JM, Colman RF and Colman RW, Low-K_m cyclic AMP phosphodiesterase from human platelets. Stimulation of activity by phosphorylation of the enzyme and affinity labeling of the active site. *Adv Second Messenger Phosphoprotein Res* **25:** 73–86, 1992.
5. Kono T, Robinson FW and Sarver JA, Insulin-sensitive phosphodiesterase. *J Biol Chem* **250:** 7826–7835, 1975.
6. Makino H and Kono T, Characterization of insulin-sensitive phosphodiesterase in fat cells. *J Biol Chem* **255:** 7850–7854, 1980.
7. Heyworth CM, Wallace AV and Houslay MD, Insulin and glucagon regulate the activation of two distinct membrane-bound cyclic AMP phosphodiesterases in hepatocytes. *Biochem J* **214:** 99–110, 1983.
8. Allan EH and Sneyd JGT, An effect of glucagon on 3′,5′-cyclic AMP phosphodiesterase activity in isolated rat hepatocytes. *Biochem Biophys Res Commun* **62:** 594–601, 1975.
9. Swinnen JV, Joseph DR and Conti M, The mRNA encoding a high-affinity cAMP phosphodiesterase is regulated by hormones and cAMP. *Proc Natl Acad Sci USA* **86:** 8197–8201, 1989.
10. Schwartz JP and Costa E, *Beta* adrenergic receptor-mediated regulation of cyclic nucleotide phosphodiesterase in C6 glioma cells: Vinblastine blockade of isoproterenol induction. *J Pharmacol Exp Ther* **212:** 569–572, 1980.
11. Torphy TJ, Zhou H-L and Cieslinski LB, Stimulation of *beta* adrenoreceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J Pharmacol Exp Ther* **263:** 1195–1205, 1992.
12. Macphee CH, Reifsnyder DH, Moore TA and Beavo JA, Intact cell and cell-free phosphorylation and concomitant activation of a low K_m, cAMP phosphodiesterase found in human platelets. *J Cyclic Nucleotide Protein Phosphor Res* **11:** 487–496, 1987.
13. Gettys TW, Vine AJ, Simonds MF and Corbin JD, Activation of the particulate low K_m phosphodiesterase of adipocytes by addition of cAMP-dependent protein kinase. *J Biol Chem* **263:** 10359–10363, 1988.
14. Swinnen JV, Tsikalas KE and Conti M, Properties and hormonal regulation of two structurally related cAMP phosphodiesterases from the rat Sertoli cell. *J Biol Chem* **266:** 18370–18377, 1991.
15. Madelian V and Shain W, Regulation of isoproterenol-induced cyclic AMP accumulation in LRM55 glial cells by phosphodiesterase. *J Pharmacol Exp Ther* **243:** 618–623, 1987.
16. Martin DL and Shain W, High affinity transport of taurine and β-alanine and low affinity transport of γ-aminobutyric acid by a single transport system in cultured glioma cells. *J Biol Chem* **254:** 7076–7084, 1979.
17. Madelian V, Martin DL, Lepore LR, Perrone M and Shain W, Beta receptor stimulated and cyclic AMP mediated taurine release from LRM55 glial cells. *J Neurosci* **5:** 3154–3160, 1985.
18. Bradford MM, 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.
19. Silver PJ, Gordon RJ, Buchholz RA, Dundore RL, Ferguson EW, Harris AL and Pagani ED, Comparative studies on cyclic nucleotide phosphodiesterases and inhibitors in experimental models of hypertension, congestive heart failure, and allergic asthma. *Adv Second Messenger Phosphoprotein Res* **25:** 341–352, 1992.
20. Ross PS, Manganiello VC and Vaughan M, Regulation of cyclic nucleotide phosphodiesterases in cultured hepatoma cells by dexamethasone and N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-monophosphate. *J Biol Chem* **252:** 1448–1452, 1977.
21. Loten EG and Sneyd JGT, An effect of insulin on adipose-tissue adenosine 3′:5′-cyclic monophosphate phosphodiesterase. *Biochem J* **120:** 187–193, 1970.
22. Houslay MD, Wallace AV, Marchmont RJ, Martin BR and Heyworth CM, Insulin controls intracellular cyclic AMP concentrations in hepatocytes by activating specific cyclic AMP phosphodiesterases: Phosphorylation of the peripheral plasma membrane enzyme. *Adv Cyclic Nucleotide Protein Phosphorylation Res* **16:** 159–176, 1984.
23. Macphee, CH, Reifsnyder DH, Moore TA, Lerea KM and Beavo JA, Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. *J Biol Chem* **263:** 10353–10358, 1988.
24. Manganiello VC, Smith CJ, Newman AH, Rice K, Degerman E and Belfrage P, Hormonal regulation of adipoxyte particulate "low K_m" cAMP phosphodiesterase. *J Cyclic Nucleotide Protein Phosphor Res* **11:** 497–511, 1987.
25. Degerman E, Smith CJ, Tornqvist H, Vasta V, Belfrage P and Manganiello VC, Evidence that insulin and isoprenaline activate the cGMP-inhibited low K_m cAMP phosphodiesterase in rat fat cells by phosphorylation. *Proc Natl Acad Sci USA* **87:** 533–537, 1990.
26. Manganiello VC, Degerman E, Smith CJ, Vasta V, Tornqvist H and Belfrage P, Mechanisms for activation of the rat adipocyte particulate cyclic-GMP-inhibited cyclic AMP phosphodiesterase and its importance in the antilipolytic action of insulin. *Adv Second Messenger Phosphoprotein Res* **25:** 147–164, 1992.
27. Sharma R and Wang J, Differential regulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by cyclic AMP-dependent protein kinase and calmodulin-dependent phosphatase. *Proc Natl Acad Sci USA* **82:** 2603–2607, 1985.
28. Sharma R and Wang J, Calmodulin and Ca^{2+}-dependent phosphorylation and dephosphorylation of 63-kDa subunit-containing bovine brain calmodulin-stimulated cyclic nucleotide phosphodiesterase isozyme. *J Biol Chem* **261:** 1322–1328, 1986.
29. Sharma R, Phosphorylation and characterization of bovine heart calmodulin-dependent phosphodiesterase. *Biochemistry* **30:** 5963–5968, 1991.
30. Welch JE, Swinnen JV, O'Brien DA, Eddy EM and Conti M, Unique adenosine 3′,5′ cyclic monophosphate phosphodiesterase messenger ribonucleic acids in rat spermatogenic cells: Evidence for differential gene expression during spermatogenesis. *Biol Reprod* **46:** 1027–1033, 1992.
31. Onali P, Schwartz JP, Hanbauer I and Costa E, Regulation by a β-adrenergic receptor of a Ca^{2+}-independent adenosine 3′,5′-(cyclic)monophosphate phosphodiesterase in C6 glioma cells. *Biochim Biophys Acta* **675:** 285–292, 1981.