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δ-Opioid Receptor Activates cAMP Phosphodiesterase Activities in Neuroblastoma × Glioma NG108–15 Hybrid Cells

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SUMMARY

In neuroblastoma × glioma NG108–15 hybrid cells, opioid agonists inhibited both basal and prostaglandin E_1-stimulated adenylate cyclase activities assayed in the presence of the phosphodiesterase (PDE) inhibitors isobutylmethylxanthine and ZK62711 (rolipram). However, when intracellular [^{3}H]cAMP was measured in the absence of the PDE inhibitors the maximal inhibitory level was increased, using the opioid agonist D-Ala^2,D-Leu^5-enkephalin. This increase in opioid activity was due to agonist stimulation of cAMP degradation, because when the degradation rate of [^{3}H]cAMP was measured in intact hybrid cells it was observed to increase from the control value of 0.495 ± 0.003 min^{-1} to 0.760 ± 0.003 min^{-1} in the presence of 1 μM D-Ala^2,D-Leu^5-enkephalin; this was reversed by naloxone. Dose-dependent studies with various opioid agonists, partial agonists, and antagonists revealed that there was a direct correlation between the abilities of these opioid ligands to inhibit adenylate cyclase activity and to stimulate PDE activity, with enkephalin and its analogs being the most potent agonists. Chronic agonist treatment also resulted in a reduction of the opioid agonist stimulation of cAMP degradation, with an apparent decrease in the PDE activity upon addition of naloxone after chronic treatment. However, treatment of the hybrid cells with pertussis toxin, which attenuated the agonist inhibition of adenylate cyclase activity, did not abolish this opioid response. When selective inhibitors for various types of PDE were used, the type I PDE inhibitor W-7 attenuated the opioid effect, whereas the type II PDE inhibitor trequinsin (HL725), the type III PDE inhibitor indolidan, and the type IV PDE inhibitor rolipram had no effect on opioid-stimulated cAMP degradation. The stimulation of type I PDE activity by δ-opioid receptors was independent of extracellular Ca^{2+} and was not observed with membrane preparations. Therefore, in NG108–15 cells δ-opioid receptors regulate intracellular cAMP levels by coupling to a pertussis toxin-insensitive guanine nucleotide-binding protein, resulting in an increase in intracellular Ca^{2+} and in Ca^{2+}/calmodulin-dependent PDE activity.

In neuroblastoma × glioma NG108–15 hybrid cells, intracellular cAMP is regulated by multiple receptors, one of which is the δ-opioid receptor. In control hybrid cells, opioid agonists such as DADLE decrease intracellular cAMP levels by inhibiting adenylate cyclase activity (1, 2). This opioid agonist inhibition of adenylate cyclase activity can be attenuated by pretreatment of the hybrid cells or membranes with PTX, which has been reported to catalyze ADP-ribosylation of the carboxyl-terminal cysteine moiety of the α subunit of several members of the G protein family (3, 4). Recent studies with specific G_α antibodies indicated that antibodies developed against $G_{i2\alpha}$-specific sequences could attenuate DADLE-stimulated low-K_m GTPase activity and DADLE inhibition of adenylate cyclase activity (5). Thus, δ-opioid receptor regulation of intracellular cAMP levels appears to be due to receptor activation of $G_{i2\alpha}$ and subsequent inhibition of adenylate cyclase activity.

In addition to coupling to $G_{i2\alpha}$, δ-opioid receptors in NG108–15 cells have been demonstrated to interact with other G proteins. Using a photoaffinity analog of GTP, GTP azidoanilanide, Offermanns *et al.* (6) reported opioid agonist-induced labeling of multiple G_α subunits. Using the ability of cholera toxin to catalyze the ADP-ribosylation of PTX substrates by NAD^+ (7), we recently demonstrated that DADLE could simultaneously induce coupling between δ-opioid receptors and three different PTX substrates, namely, $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$ (8). Hence, it is likely that activation of the δ-opioid receptor in NG108–15 hybrid cells can elicit multiple cellular responses.

The ability of opioid agonists to regulate cellular effectors other than adenylate cyclase has been reported. In differentiated NG108–15 cells, DADLE regulates voltage-dependent Ca^{2+} channels via interaction with G_o (9) and regulates the Na^+/H^+ exchanger via interaction with a non-PTX substrate (10). However, activation of effectors other than adenylate

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ABBREVIATIONS: DADLE, D-Ala^2,D-Leu^5-enkephalin; PDE, phosphodiesterase; PTX, pertussis toxin; G protein, guanine nucleotide-binding protein; PGE_1, prostaglandin E_1; IBMX, isobutylmethylxanthine; KRHB, Krebs-Ringer HEPES buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

cyclase by a δ-opioid agonist in nondifferentiated hybrid cells has not yet been reported. Because one of the G proteins that could be activated by the opioid receptor is $G_{i3\alpha}$, and $G_{i3\alpha}$ has been implicated in the control of phospholipase C in neutrophils (4), opioid agonists may regulate cellular enzymes that are dependent on the intracellular Ca^{2+} level. One such enzyme is the Ca^{2+}/calmodulin-dependent PDE (type I PDE).

Some findings suggest that G protein-coupled receptors can regulate PDE activity. Muscarinic receptors have been shown to decrease intracellular cAMP levels in astrocytoma cells by stimulating PDE activity (11). Chronic muscarinic receptor activation resulted in an elevation of intracellular cAMP levels in NG108–15 cells, which could be accounted for partially by a decrease in the cAMP degradation rate (12). The notion that opioid agonists, like muscarinic agonists, can activate PDE activity is supported by the observation that acute opioid agonist treatment caused the translocation of calmodulin to the plasma membrane (13). Because calmodulin can activate type I PDE, it is probable that δ-opioid receptors can stimulate PDE activity. In this way, the intracellular cAMP level in NG108–15 cells would be regulated by opioid agonist control of both synthesis and degradation rates for the cyclic nucleotide. Therefore, in the present report we have attempted to demonstrate the activation of PDE by δ-opioid receptors in NG108–15 cells. By using selective PDE inhibitors, we were able to demonstrate that δ-opioid receptor activation resulted in the stimulation of type I PDE activity in the hybrid cells.

Materials and Methods

Culturing of NG108–15 hybrid cells. The initial stock culture of NG108–15 cells was obtained from Dr. B. Hamprecht (Physiologischemishes Institut des Universitat, Wurzburg, Germany). Briefly, NG108–15 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 0.1 mM hypoxanthine, 0.1 μM aminopterin, 16 μM thymidine, and 5% heat-inactivated fetal calf serum, in a 10% CO_2 atmosphere at 37°. For the PDE activity measurements, hybrid cells were seeded at 42,000 cells/well in 24-well cluster tissue culture plates (Costar, Cambridge, MA) 4 days before experiments. The growth medium was changed completely 1 day before experiments.

Measurement of opioid agonist inhibition of intracellular cAMP levels. Opioid agonist inhibition of intracellular production was determined by measuring, in the presence of various concentrations of PGE_1 and opioid agonists, the [^{3}H]cAMP formed from intracellular ATP pools that had been prelabeled with [^{3}H]adenine (1). The hybrid cells were cultured in 17-mm plates in standard growth medium. On the day of the experiment, the media were removed, 0.5 ml of incubation medium containing 2.5 μCi of [^{3}H]adenine and 0.25 mM IBMX was added, and the cells were incubated in 10% CO_2 at 37° for 1 hr. Radioactive media were then removed and 0.5 ml of KRHB, pH 7.4, containing various concentrations of PGE_1 and opioid agonists was added to the plates. When PDE inhibitors were added, 0.25 mM IBMX and 0.1 mM rolipram were introduced into the cells during [^{3}H]adenine labeling and were kept in the incubation medium upon the addition of PGE_1 and opioid agonists. Incubations were carried out for 10 min at 37° and then terminated by the addition of 0.05 ml of 3.3 N perchloric acid. [^{3}H]cAMP was then separated from other radioactive nucleotides by Dowex and alumina column chromatography, as described by White and Karr (14).

Measurement of PDE activity. The PDE activity of NG108–15 cells was measured by determining the degradation of elevated levels of [^{3}H]cAMP in intact hybrid cells. Hybrid cells were prelabeled with [^{3}H]adenine (50 μCi/10 ml) by the complete removal of growth medium and replacement with 0.5 ml of incubation medium (Dulbecco's modified Eagle's medium without serum plus 0.45 g of NaCl/500 ml). The hybrid cells were then incubated for 1 hr at 37° under a 10% CO_2 atmosphere. The labeling mixture was then aspirated, 0.5 ml of KRHB, pH 7.4, containing 10 μM PGE_1 was added, and the cells were incubated at 37° for 10 min. The stimulation of adenylate cyclase activity by PGE_1 was terminated by placing the 24-well plates on ice and removing the KRHB containing PGE_1. Excess PGE_1 was then removed by washing the hybrid cells twice with 0.5 ml of KRHB at 4°. Reaction mixtures containing various opioid ligands at 4° were then added to the appropriate wells, and the 24-well plates were then incubated again at 37°. At the desired time the degradation was terminated by the addition of 50 μl of 3.3 N perchloric acid, and [^{3}H]cAMP was separated from other radioactive nucleotides by the double-column chromatography technique (14). The degradation rate for cAMP was then calculated by plotting the natural logarithm of the ratio between the initial [^{3}H]cAMP level (A_o) and the [^{3}H]cAMP level at any time (A) versus time.

When membrane PDE activities were measured, the assay method described by Thompson *et al.* (15) was followed. Briefly, membrane pellets (100,000 × *g* for 1 hr), minus nuclei, isolated from NG108–15 cells were resuspended in assay buffer (40 mM Tris, pH 8.0, 4 mM β-mercaptoethanol). To each assay mixture (final volume, 0.4 ml), 0.1 μM [^{3}H]cAMP (0.5 μCi), 5 mM $MgCl_2$, and 100 μg of protein were added. The incubation was carried out at 30° for various times. At the appropriate time, 0.15 ml of 1 N $HClO_4$ was added to terminate the reaction. The [^{3}H]cAMP remaining was then determined by separating the cyclic nucleotide from other nucleotides by a Dowex and alumina chromatographic procedure, after the addition of 10,000 cpm of standard [^{32}P]cAMP (14).

Materials. [^{3}H]cAMP (40 Ci/mmol) and [^{3}H]adenine (16 Ci/mmol) were supplied by New England Nuclear (Boston, MA). Peninsula Laboratories (Belmont, CA) supplied all the opioid peptides used. Rolipram (ZK62711) and indolidan (LY195115) were generous gifts from Schering (Berlin, Germany) and Eli Lily (Indianapolis, IN), respectively. HL-725 and W-7 were purchased from Calbiochem (La Jolla, CA). All other reagents were supplied by Sigma Chemical Co. (St. Louis, MO).

Results

Comparison of opioid inhibition in the presence and absence of PDE inhibitors. In our routine assays of opioid agonist regulation of intracellular cAMP levels, two PDE inhibitors, IBMX and rolipram (ZK62711), were added to the incubation mixtures. When the dose-response curve for the opioid agonist DADLE attenuating the PGE_1 (5 μM)-stimulated increase in intracellular cAMP levels in NG108–15 was determined, the agonist was observed to inhibit this process by 68.4 ± 4.9%, with an IC_{50} value of 0.95 ± 0.21 nM (Fig. 1). When parallel assays were carried out in the absence of the two PDE inhibitors, the amount of intracellular [^{3}H]cAMP produced after 10 min of incubation in the presence of 5 μM PGE_1 was drastically reduced, as expected. PGE_1 at 5 μM stimulated the production of intracellular [^{3}H]cAMP to the level of 16,200 ± 280 cpm/mg of protein/10 min in the absence of PDE inhibitors, whereas the level of [^{3}H]cAMP reached 108,900 ± 5,030 cpm/mg of protein/10 min in the presence of the PDE inhibitors. Nevertheless, the opioid agonist appeared to inhibit the production of intracellular [^{3}H]cAMP to a greater extent. In the absence of PDE inhibitors, PGE_1-stimulated production of [^{3}H]cAMP was maximally inhibited 88.2 ± 0.3% by DADLE. The IC_{50} value of the agonist was determined to be 1.6 ± 0.8 nM, which was not significantly different from the IC_{50} value obtained in the presence of PDE inhibitors. Thus, the opioid agonist DADLE appeared to regulate the intracellular [^{3}H]

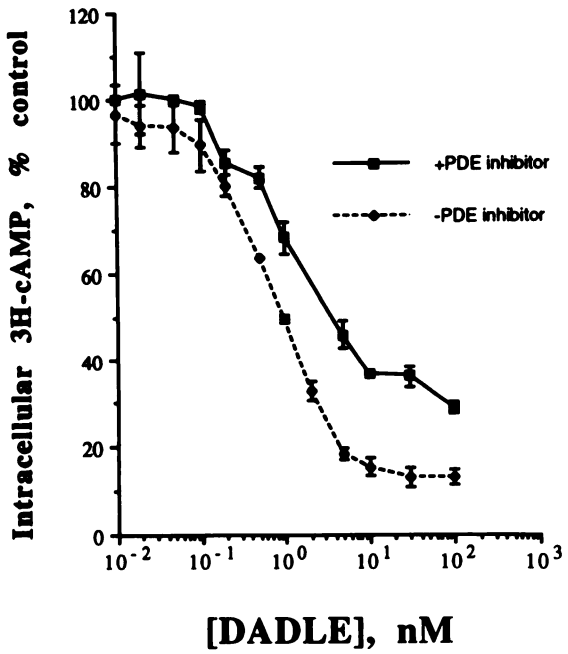

Fig. 1. DADLE concentration-dependent inhibition of PGE_1-stimulated intracellular [^{3}H]cAMP production in NG108–15 cells. NG108–15 cells were cultured and labeled with [^{3}H]adenine as described in Materials and Methods. The ability of various concentrations of DADLE to inhibit 5 μM PGE_1-stimulated production of [^{3}H]cAMP at 37° for 10 min was then determined in the presence (⊡) or in the absence (♦) of 0.25 mM IBMX and 0.1 mM rolipram. The values represent averages of determinations from three separate plates.

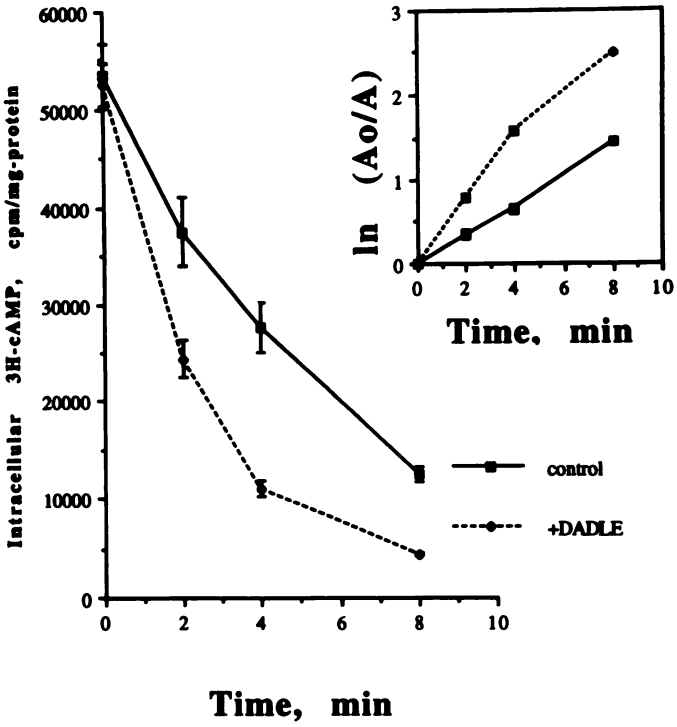

Fig. 2. Time-dependent decrease in intracellular [^{3}H]cAMP levels in NG108–15 cells. Intracellular [^{3}H]cAMP levels were elevated by incubating NG108–15 cells in the presence of 5 μM PGE_1 at 37° for 10 min. After repeated washing to remove PGE_1, the degradation of [^{3}H]cAMP formed was measured by incubating NG108–15 cells at 37° in the presence (♦) or in the absence (⊡) of 100 nM DADLE. Incubations were terminated at various times and the levels of cAMP remaining were determined. *Inset*, natural logarithm of the ratio of the level of cAMP at time 0 to that when incubation was terminated. The values represent averages of determinations from three separate plates.

cAMP level via a pathway other than the well studied agonist inhibition of adenylate cyclase activity.

Opioid agonist stimulation of [^{3}H]cAMP degradation. The apparent difference in the maximal level of intracellular [^{3}H]cAMP produced in the presence of agonist and in the presence or absence of PDE inhibitors could be due to the stimulation of PDE activity by the opioid agonist. If this is the case, then the rate of [^{3}H]cAMP degradation should be greater in the presence of agonist. When the intracellular [^{3}H]cAMP level in NG108–15 hybrid cells was elevated with PGE_1 and the stimulator was then removed by repeated washing, there was a time-dependent decrease in intracellular [^{3}H]cAMP when the hybrid cells were incubated at 37° (Fig. 2). The basal rate of the [^{3}H]cAMP degradation was determined to be 0.495 ± 0.003 min^{-1}. In the presence of 100 nM DADLE, the rate of [^{3}H]cAMP degradation was observed to increase by 70%, to 0.760 ± 0.003 min^{-1}. Because it was observed that the PDE activity was linear within 5 min of incubation, in subsequent experiments the PDE activity in intact NG108–15 cells was determined within the 5-min period.

The DADLE-stimulated PDE activity was due to activation of δ-opioid receptors present in the hybrid cells. When the ability of various concentrations of DADLE to increase the [^{3}H]cAMP degradation was measured and the rates were determined, it was observed that the EC_{50} value of DADLE to stimulate the [^{3}H]cAMP degradation was 0.86 ± 0.22 nM (Fig. 3). This value is similar to the IC_{50} value of DADLE to inhibit production of intracellular [^{3}H]cAMP (Fig. 1). Furthermore, when the dose-response curve for DADLE was determined in the presence of 1 μM naloxone, a parallel rightward shift of the dose-response curve was observed (Fig. 3), with the EC_{50} value of DADLE being increased to 10.6 ± 1.6 nM. When the relative potencies of various opioid ligands to increase the rate of [^{3}H]cAMP degradation in NG108–15 cells were determined, it was observed that this opioid effect followed the δ-opioid receptor inhibition of adenylate cyclase activity, i.e., opioid ligands that are potent in their inhibition of adenylate cyclase activity also are potent in their stimulation of [^{3}H]cAMP degradation. As summarized in Table 1, opioid ligands that are derivatives of the pentapeptide enkephalin and that are selective for the δ-opioid receptor, e.g., DADLE and D-Ala^2,Met^5-enkephalin, were the most potent in their stimulation of [^{3}H]cAMP production. Opioid ligands that are partial agonists in the δ-opioid receptor system in NG108–15 cells, e.g., diprenorphine, levorphanol, and morphine, were also partial agonists in the stimulation of [^{3}H]cAMP degradation. Opioid ligands that are μ-opioid receptor selective and that do not inhibit adenylate cyclase activity, e.g., morphiceptin (Tyr-Pro-Phe-Pro-amide), also did not stimulate [^{3}H]cAMP degradation. Thus, it is apparent that opioid ligand stimulation of [^{3}H]cAMP degradation in NG108–15 cells is mediated by the δ-opioid receptor present in these cells.

It was possible that the increase in the rate of [^{3}H]cAMP degradation in the presence of opioid agonists was simply due to inhibition of [^{3}H]cAMP production. To elevate the intracellular [^{3}H]cAMP level, 5 μM PGE_1 was added to the incubation medium. Although the stimulator was removed by washing, an increase in the intracellular [^{3}H]cAMP production might persist after removal of PGE_1. However, this was not the case, inasmuch as the presence of PDE inhibitors did not cause a

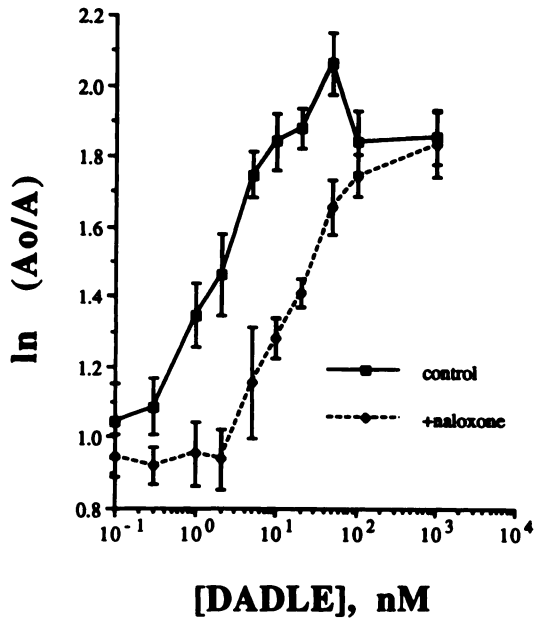

Fig. 3. DADLE concentration-dependent stimulation of [^{3}H]cAMP degradation in NG108–15 cells in the absence (□) and in the presence (♦) of 1 μM naloxone. NG108–15 cells were prelabeled with [^{3}H]adenine and the intracellular [^{3}H]cAMP level was elevated with PGE_1 as described in Materials and Methods. The amount of [^{3}H]cAMP degraded after 4 min of incubation at 37° was then determined in the presence of various concentrations of DADLE, with or without naloxone. The values represent averages of determinations from three separate plates.

TABLE 1

Relative potencies of various opioid ligands to decrease intracellular [^{3}H]-cAMP accumulation and to stimulate PDE activities in NG108-15 hybrid cells

Measurements of intracellular [^{3}H]-cAMP levels and PDE activities were carried out as described in Materials and Methods. The opioid ligand concentration-dependent modulation of the enzyme activities was determined with at least 12 different concentrations of opioid ligands. Intrinsic activities (IA) of the ligands were determined by comparing the maximal activity of the ligand with that of etorphine, which has an intrinsic activity of 1. The values of K_i for decreasing the intracellular [^{3}H]-cAMP levels, measured in the presence of the PDE inhibitors IBMX and rolipram (with the exception of α-neoendorphin), were from Ref. 32.

Opioid ligands	IA	K_{act} (PDE)	K_i
		nM	nM
D-Ala^2,Met^5-Enkephalin	0.98	0.18 ± 0.08	0.89 ± 0.27
DADLE	0.92	0.86 ± 0.22	2.1 ± 0.2
D-Ala^2,D-Met^5-Enkephalin	0.91	2.6 ± 0.7	2.4 ± 0.6
Etorphine	1.00	1.6 ± 0.6	2.2 ± 0.7
Diprenorphine	0.70	7.1 ± 2.3	2.1 ± 0.3
α-Neoendorphin	0.97	5.9 ± 2.5	11.7 ± 0.19
β_h-Endorphin	0.99	59 ± 20	95 ± 22
Dynorphin-1-13	0.96	78 ± 16	86 ± 18
Levorphanol	0.65	550 ± 190	150 ± 11
Morphine	0.69	7,900 ± 2,500	3,400 ± 290
Naloxone	0	88 ± 13	46 ± 5.8
Tyr-Pro-Phe-Pro-amide	0	>10,000	>10,000

continuing increase in intracellular [^{3}H]cAMP levels after the removal of PGE_1. Furthermore, as seen in later studies with different PDE inhibitors, it was possible to attenuate opioid-stimulated PDE without altering basal PDE activities (see Fig. 10). Hence, the observed increase in the degradation of intracellular [^{3}H]cAMP was due to opioid agonist stimulation of PDE activity.

Effect of PTX on opioid attenuation of intracellular [^{3}H]cAMP levels. δ-Opioid receptors inhibit intracellular cAMP production in NG108–15 cells via the activation of a specific class of G proteins, mostly likely G_{i2} (5). Pretreatment of the hybrid cells with PTX attenuates opioid agonist inhibition of the adenylate cyclase activity (3). To examine whether the observed opioid agonist stimulation of the PDE activity was mediated via the same G protein family, the effect of PTX on the agonist activity was determined. NG108–15 cells were pretreated with 100 ng/ml PTX at 37° for 3 hr. As shown in Fig. 4, this paradigm of PTX treatment ADP-ribosylated >90% of the G_α subunits, which are PTX substrates. Membranes isolated from NG108–15 cells thus treated exhibited >90% reduction, compared with membranes isolated from control NG108–15 cells, in [^{32}P]ADP-ribose incorporated into proteins of 39–42 kDa (Fig. 4). During the last hour of treatment the cells were labeled with [^{3}H]adenine and the intracellular [^{3}H]cAMP level was elevated with various concentrations of PGE_1 in the presence or absence of 100 nM DADLE. As shown in Fig. 5, in the presence of the PDE inhibitors IBMX and rolipram there was a PGE_1-dependent increase in the intracellular [^{3}H]cAMP level, with a maximal stimulation of 36-fold at 10 μM PGE_1. In the presence of 100 nM DADLE the PGE_1 stimulation was attenuated. At 10 nM PGE_1 DADLE inhibited the stimulation by 95 ± 0.6%, whereas at 10 μM PGE_1 DADLE inhibited stimulation by 66 ± 0.9%. This observed progressive decrease in the DADLE inhibition level could be due to direct competition of a constant level of activated G_i generated by DADLE-occupied δ-opioid receptors with increasing levels of activated G_s generated by increasing numbers of occupied PGE_1 receptors. Nonetheless, pretreatment of the hybrid cells with PTX reduced the ability of DADLE to inhibit PGE_1-stimulated

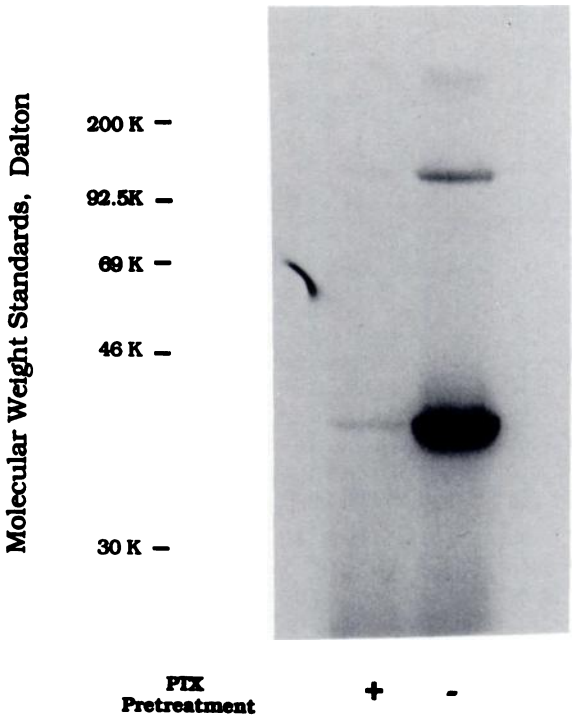

Fig. 4. PTX-catalyzed [^{32}P]ADP-ribosylation of NG108–15 membrane proteins by [^{32}P]NAD^+. NG108–15 hybrid cells were pretreated with 100 ng/ml PTX at 37° for 3 hr. P_2/P_3 membranes were then isolated and PTX-catalyzed ADP-ribosylation with [^{32}P]NAD^+ was carried out as described (8). Parallel labeling experiments were carried out with membranes isolated from control cells. The membrane proteins were then solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography were carried out.

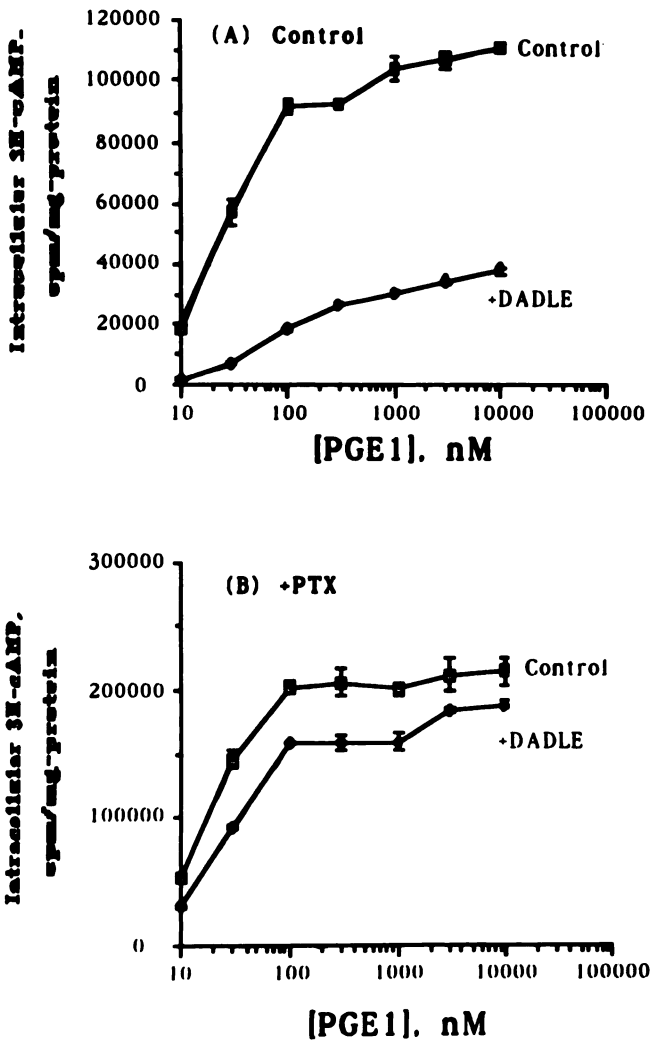

Fig. 5. PGE_1 concentration-dependent stimulation of [^{3}H]cAMP accumulation in NG108–15 cells in the presence of the PDE inhibitors IBMX and rolipram. A, Intracellular [^{3}H]cAMP accumulation at various concentrations of PGE_1 in the absence (□) and in the presence (♦) of 100 nM DADLE. B, NG108–15 cells were treated with 100 ng/ml PTX at 37° for 3 hr before the [^{3}H]cAMP accumulation at various concentrations of PGE_1 in the absence (□) and in the presence (♦) of 100 nM DADLE was determined. The values represent averages from three separate plates.

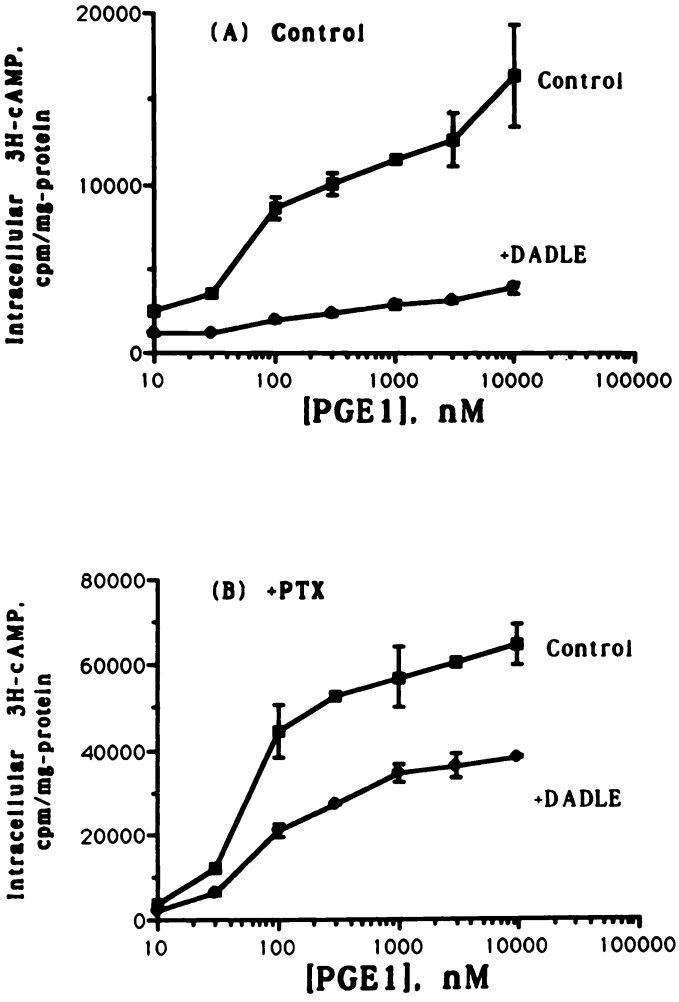

Fig. 6. PGE_1 concentration-dependent stimulation of [^{3}H]cAMP accumulation in NG108–15 cells in the absence of PDE inhibitors. The experiments were carried out in parallel with those summarized in Fig. 5, using the same passage of hybrid cells. Intracellular [^{3}H]cAMP was measured in the absence (□) and in the presence (♦) of 100 nM DADLE, with control hybrid cells (A) or with hybrid cells treated with PTX (100 ng/ml for 3 hr at 37°) (B). The values represent averages from three separate plates.

intracellular [^{3}H]cAMP production. As shown in Fig. 5, PTX treatment increased the intracellular level of [^{3}H]cAMP (1.9-fold increase). At 10 nM PGE_1, 100 nM DADLE inhibited the production of [^{3}H]cAMP by 41 ± 2.1%. At 10 μM PGE_1, after PTX pretreatment, 100 nM DADLE inhibited the production of [^{3}H]cAMP by 10 ± 1.1%. Thus, as expected, PTX pretreatment under the current paradigm reduced opioid agonist inhibition of adenylate cyclase activity.

On the other hand, when the NG108–15 hybrid cells were pretreated with PTX and then the ability of various concentrations of PGE_1 to stimulate intracellular [^{3}H]cAMP production in the presence or absence of DADLE was monitored in the absence of any PDE inhibitors, a pattern different from those obtained in the presence of PDE inhibitors was observed. As shown in Fig. 6, in the absence of any PDE inhibitors 100 nM DADLE inhibited 10 nM PGE_1-stimulated production of intracellular [^{3}H]cAMP by 55 ± 1.4% and 10 μM PGE_1-stimulated production by 77 ± 1.2%. After PTX pretreatment, opioid inhibition of intracellular [^{3}H]cAMP production in the absence of PDE inhibitors was also attenuated (Fig. 6). After PTX treatment, 100 nM DADLE inhibited 10 nM PGE_1 stimulation by 46 ± 0.6% and inhibited 10 μM PGE_1 stimulation by 41 ± 0.3%. The opioid agonist attenuation of intracellular [^{3}H]cAMP production apparently was not dependent on the percentage of PGE_1 receptors being occupied. Because in the presence of PDE inhibitors, at 10 μM PGE_1, DADLE attenuated the intracellular [^{3}H]cAMP level by 10% (Fig. 5), it can be concluded that the major component of the 40% inhibition of intracellular [^{3}H]cAMP production by the opioid agonist after PTX pretreatment was not due to inhibition of adenylate cyclase activity; rather, it was due to stimulation of PDE activity. This opioid receptor stimulation of PDE activity was not affected by PTX pretreatment. The insensitivity of this DADLE effect was demonstrated by direct measurement of [^{3}H]cAMP decay in control NG108–15 cells and hybrid cells pretreated with PTX. As shown in Fig. 7, PTX pretreatment appeared to stimulate PDE activity. In the presence of 100 nM DADLE,

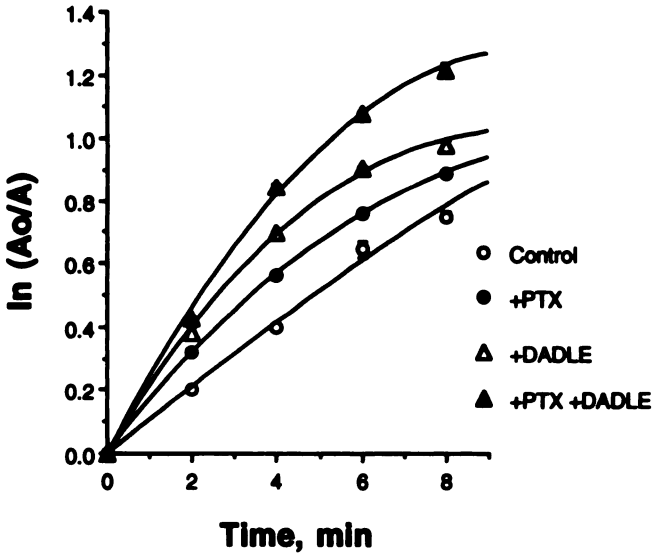

Fig. 7. Effect of PTX pretreatment on PDE activity in NG108–15 cells. Hybrid cells were pretreated with 100 ng/ml PTX for 24 hr at 37°. The hybrid cells were then labeled with [^{3}H]adenine and the intracellular [^{3}H]cAMP level was elevated with PGE_1 as described in Materials and Methods. The ability of 100 nM DADLE to stimulate PDE activity in control cells (△) or PTX-pretreated cells (▲) was then compared with the PDE activity in control (○) or PTX-pretreated (●) cells in the presence of saline.

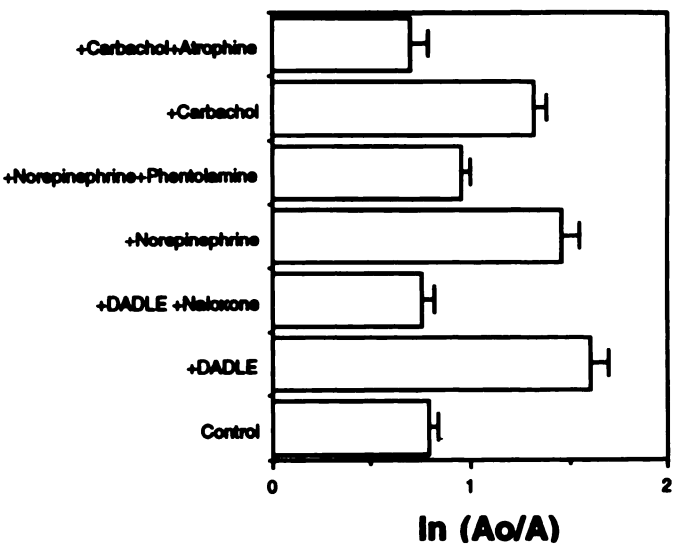

Fig. 8. Stimulation of PDE activity by various receptors in NG108–15 cells. The amount of [^{3}H]cAMP degraded in 4-min incubations of NG108–15 cells at 37° was measured in the presence of various receptor agonists and antagonists. For the muscarinic receptor agonist/antagonist pair carbachol/atropine, 10 μM concentrations of the drugs were used in the assays. For the α_2-adrenergic receptor pair norepinephrine/phentolamine, 10 μM and 50 μM, respectively, were used. For the opioid receptor pair DADLE/naloxone, 100 nM and 100 μM, respectively, were used. The values represent averages from three separate plates.

the rate of [^{3}H]cAMP degradation was further increased in PTX-treated cells.

Some characteristics of opioid agonist stimulation of [^{3}H]cAMP degradation. From the studies described thus far, it could be concluded that the opioid agonist regulates intracellular cAMP levels in NG108–15 cells by both inhibiting the synthesis (adenylate cyclase) and stimulating the degradation (PDE) of the cyclic nucleotide. The ability of other inhibitory receptor agonists, such as carbachol (M_4 muscarinic receptor agonist) and norepinephrine [α_2 (C10)-adrenergic receptor agonist], to stimulate intracellular [^{3}H]cAMP degradation was also observed (Fig. 8). In the presence of either 10 μM carbachol or norepinephrine, the PDE activities were increased. The levels of the PDE activity increases in the presence of these two agonists were similar, compared with that seen in the presence of DADLE (Fig. 8). Furthermore, carbachol- and norepinephrine-stimulated PDE activities could be antagonized by 10 μM atrophine and phentolamine, respectively. Thus, it appears that these inhibitory receptors, like δ-opioid receptors, can regulate intracellular cAMP levels via the activation of PDE.

To determine whether the opioid receptor-mediated stimulation of [^{3}H]cAMP degradation was the result of a direct interaction of the δ-opioid receptor with PDE, the PDE activity in a membrane preparation was assayed. When P_2 membranes of NG108–15 cells were prepared and PDE activities were measured, there was no apparent difference in the enzymatic activities in the presence or absence of DADLE (Fig. 9). The absence of any opioid effect was observed at various concentrations of membrane preparations and at various [^{3}H]cAMP concentrations. When the enzymatic parameters of PDE associated with the P_2 membranes of NG108–15 cells were determined, the K_m value for cAMP and the V_{max} value for cAMP hydrolysis were found to be similar in the presence and absence of DADLE (Fig. 9). The K_m values were 0.79 μM and 0.76 μM

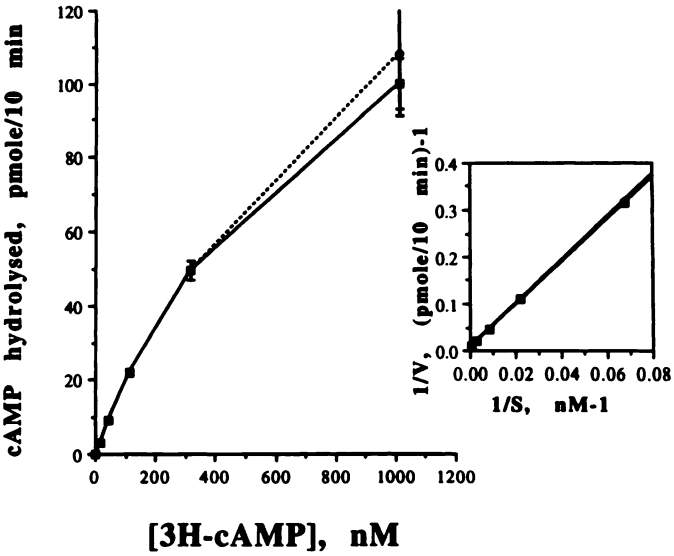

Fig. 9. [^{3}H]cAMP concentration dependence of the PDE activity in NG108–15 cell membranes in the absence (□) or in the presence (◆) of 100 nM DADLE. Membranes from NG108–15 cells, homogenized in 40 mM Tris, pH 8.0, 4 mM mercaptoethanol, were isolated by centrifugation of the homogenate at 22,000 × g for 30 min. The pellets were washed once with the same buffer and used for the PDE assays as described in Materials and Methods. One hundred-fifty micrograms of protein were used in each assay. *Inset*, Lineweaver-Burke plot of the PDE activity determined with these concentrations of cAMP.

and the V_{max} values were 0.170 nmol/10 min and 0.167 nmol/10 min in the absence and in the presence of 100 nM DADLE, respectively. Therefore, unlike the case in whole cells, opioid agonist did not regulate the PDE activities associated with the P_2 membranes.

Subsequently, the question of whether opioid agonist stimulation of [^{3}H]cAMP degradation could be desensitized was

examined. NG108–15 hybrid cells were treated with 100 nM DADLE for 24 hr and then labeled with [^{3}H]adenine, still in the presence of DADLE to prevent the cells from undergoing spontaneous reversal. The intracellular [^{3}H]cAMP level was then elevated with 10 μM PGE_1 and degradation of the cyclic nucleotide was measured in the presence or absence of the opioid agonist DADLE or the antagonist naloxone. Fig. 9 summarizes the data from three separate passages of chronic DADLE-treated hybrid cells. As with receptor-mediated inhibition of adenylate cyclase activity, chronic opioid agonist treatment eliminated the ability of DADLE to stimulate PDE. This desensitization was homologous, because the ability of the muscarinic agonist carbachol or the α_2-adrenergic agonist norepinephrine to stimulate PDE activity was unaltered (data not shown). Interestingly, although the difference was not statistically significant, when [^{3}H]cAMP degradation was measured in the presence of naloxone there appeared to be a decrease in PDE activity after chronic DADLE treatment (Fig. 10). This reduction in PDE activity could contribute to the overall increase in the intracellular cAMP level in NG108–15 cells after chronic agonist treatment.

Effect of PDE inhibitors on opioid agonist stimulation of [^{3}H]cAMP degradation. Based on molecular cloning and purification studies, the isozymes of PDE are grouped into five major classes, (a) type I, Ca^{2+}/calmodulin-activatable PDE; (b) type II, cGMP-activatable PDE; (c) type III, cGMP-inhibitable cAMP PDE; (d) type IV, cAMP-specific PDE; and (e) cGMP-specific PDE (16). These PDE activities can be blocked with selective inhibitors. Therefore, to elucidate the mechanism of opioid agonist-stimulated [^{3}H]cAMP degradation in NG108–15 cells the effect of opioid was measured in the presence of various PDE inhibitors. The PDE inhibitors W-7, trequinsin (HL 725), indolidan (LY 195115), and rolipram (ZK 62711) were used to selectively inhibit type I, II, III, and IV PDEs, respectively. As shown in Fig. 11, all of the PDE inhibitors except W-7 exhibited dose-dependent inhibition of cAMP degradation in the current paradigm. The DADLE-induced activity was determined by subtracting the basal PDE activity from the measured PDE activity in the presence of DADLE. With two of the inhibitors used, HL-725 and rolipram, there was also an apparent decrease in the DADLE-induced PDE activity. However, this apparent attenuation of opioid activity was due to an increase in intracellular cAMP accumulation at these concentrations of PDE inhibitors. With 1 mM HL725, 0.2 mM indolidan, and 0.01 mM rolipram, the highest concentrations of these inhibitors tested, the DADLE-induced PDE activities were observed to be 98.7 ± 16.7%, 100.6 ± 7.3%, and 97.4 ± 4.7%, respectively, compared with control (when DADLE-induced PDE activity was measured in the absence of PDE inhibitors). Hence, the type II, type III, and type IV PDE inhibitors did not alter opioid receptor-mediated acceleration of cAMP degradation.

On the other hand, when the type I PDE inhibitor W-7 was used an attenuation of opioid-stimulated PDE activity was observed. As shown in Fig. 11, only at very high concentrations of W-7 (>0.2 mM) was there a decrease in the opioid stimulation. At the highest concentrations of W-7 tested (1 mM), instead of a further decrease in the PDE activity there was an apparent increase in the PDE activity. This apparent increase was observed in both experiments. Furthermore, when DADLE-induced PDE activity with 1 mM W-7 was compared with that of control, it was found that 76.9 ± 15% of the opioid-induced activity was attenuated by this type I PDE inhibitor.

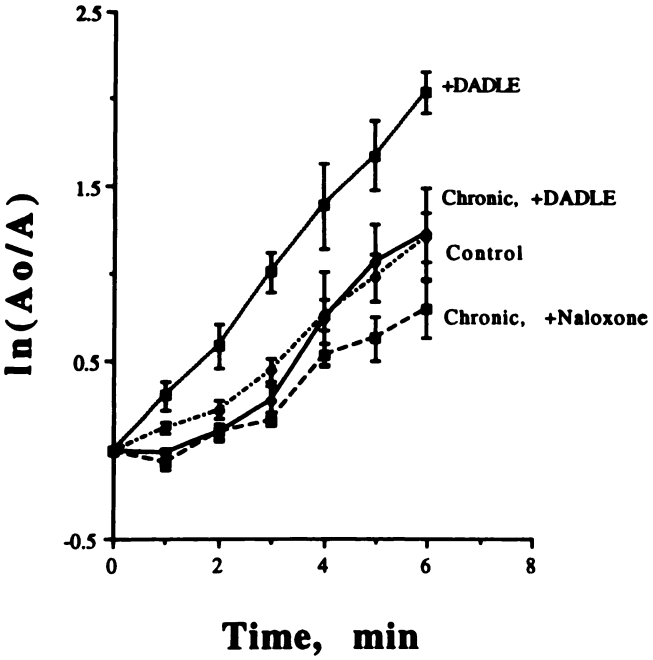

Fig. 10. Effect of chronic DADLE treatment on the opioid stimulation of PDE activity in NG108–15 cells. Hybrid cells were treated with 100 nM DADLE for 24 hr before labeling of the cells with [^{3}H]adenine and stimulation of [^{3}H]cAMP production with 5 μM PGE_1. [^{3}H]cAMP degradation was carried out in the presence of 100 nM DADLE in control cells (■) and in cells treated chronically with DADLE (◆). [^{3}H]cAMP degradation was also determined in the presence of 20 μM naloxone in control cells (◇) and in chronically DADLE-treated cells (□). The values represent averages from three separate passages of cells and three separate plates for each passage of cells.

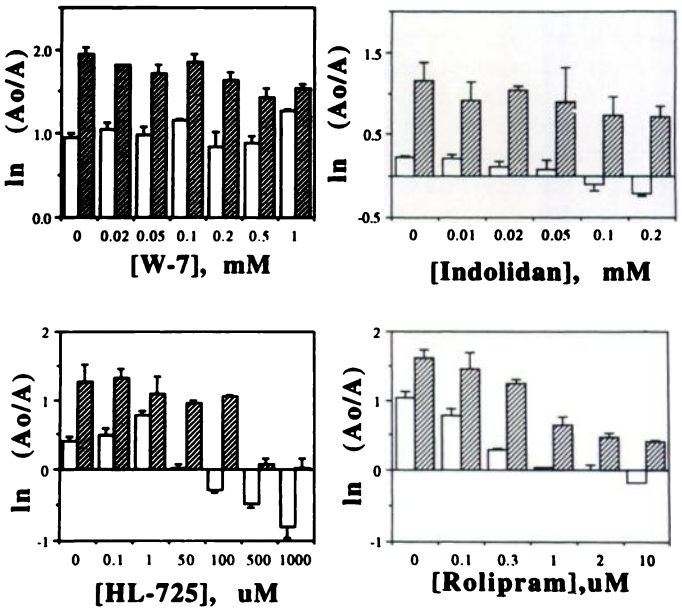

Fig. 11. Effect of various PDE inhibitors on the rate of [^{3}H]cAMP degradation in the absence (□) and in the presence (■) of 100 nM DADLE. NG108–15 cells were labeled with [^{3}H]adenine and [^{3}H]cAMP production was stimulated with 5 μM PGE_1 as described in Materials and Methods. After the removal of PGE_1, the degradation of [^{3}H]cAMP in 5 min was determined in the presence of various concentrations of PDE inhibitors. The values represent averages of determinations from three separate plates.

Such a high concentration of W-7 was needed to observe the attenuation because of the relatively low potency of this inhibitor in binding to calmodulin (17). Higher concentrations of W-7 were not tested due to solubility problems in our assays.

Evidence that opioid-stimulated PDE activity was independent of extracellular Ca^{2+}. Because the type I PDE inhibitor W-7 attenuated opioid-stimulated PDE activity in NG108–15 cells, it is possible that opioid stimulation could be due to an increase in the intracellular Ca^{2+} concentration. Recent studies reported by Okajima and Konda (18) and Jin *et al.* (19) suggested that DADLE could increase intracellular free Ca^{2+} levels independently of the extracellular Ca^{2+} level. Thus, the effect of extracellular Ca^{2+} on PDE activity in NG108–15 cells was investigated. After radioactive labeling of the intracellular ATP pools with [^{3}H]adenine and elevation of the intracellular [^{3}H]cAMP level with PGE_1, the hybrid cells were washed with KRHB without calcium in the presence of 1 mM EGTA. Care was taken not to detach the cells from the growing surface. Either KRHB containing 1 mM Ca^{2+} or KRHB without Ca^{2+} was then added to the Petri dishes, and the rate of [^{3}H]cAMP disappearance was measured. As shown in Fig. 12, the level of extracellular Ca^{2+} did not affect basal PDE activity. Furthermore, the PDE activity in the presence of DADLE was not affected by the absence of extracellular Ca^{2+}. Therefore, the apparent activation of the type I PDE activity was not due to an increase in intracellular Ca^{2+} levels produced by the opening of Ca^{2+} channels.

Discussion

The ability of opioid agonists to attenuate intracellular cAMP levels by inhibiting the synthesis of cAMP has been demonstrated unequivocally in brain and in NG108–15 cells (1, 2, 20, 21). Opioid receptor-mediated inhibition of adenylate cyclase activity has been shown to involve one of the PTX substrates, most likely $G_{i2\alpha}$ (5). In the current studies, we have demonstrated that an opioid agonist regulates intracellular cAMP levels via a second mechanism, stimulation of the cAMP-degradative enzyme PDE. Opioid activity was observed when the decay of PGE_1-stimulated intracellular cAMP levels was measured in the presence of agonist. We have shown that this opioid agonist stimulation of PDE activity is mediated via the homogeneous population of δ-opioid receptors in this cell line, the same receptors that inhibit adenylate cyclase activity.

One could argue that the observed increase in the cAMP degradation rate was due to inhibition of cAMP synthesis in the presence of the opioid agonist and not due to the increase in PDE activity. However, three sets of observations suggested otherwise, (a) the increase in maximal opioid inhibition of intracellular cAMP production in the absence of PDE inhibitors (Fig. 1), (b) the observed PDE activity in PTX-treated cells (Fig. 6), and (c) the ability of the type I PDE inhibitor W-7 to inhibit the opioid-stimulated PDE activity (Fig. 11). The maximal level of opioid inhibition was increased in the absence of PDE inhibitors, implying that the opioid agonist might increase PDE activity. Furthermore, when opioid inhibition of intracellular [^{3}H]cAMP production was determined in the presence of PDE inhibitors, it was observed that the magnitude of opioid inhibition was dependent on the concentration of stimulator, PGE_1, used in the assay (Fig. 6). This is to be expected, because regardless of the mechanism by which adenylate cyclase activity is inhibited, whether it is due to competition of $G_{i\alpha}$ and $G_{s\alpha}$ for the catalytic subunit or to the $\beta\gamma$ subunit causing reassociation of $G_{s\alpha}$ into the heterotrimeric form (22), increasing concentrations of PGE_1 will result in increasing $G_{s\alpha}$ subunit concentrations. Higher concentrations of $G_{i\alpha}$ or $\beta\gamma$ subunits will then be needed to antagonize the $G_{s\alpha}$ activity. This has been demonstrated clearly in human platelet membranes, in which $G_{i\alpha}$ subunit concentration-dependent modulation of adenylate cyclase activity was dependent on epinephrine activation of G_s (23). Because the opioid agonist stimulation of PDE activity did not exhibit a PGE_1 concentration-dependent decrease of activity in NG108–15 hybrid cells pretreated with PTX (Fig. 6), it is apparant that the opioid effect on PDE activity is distinct from opioid inhibition of adenylate cyclase activity.

When the question of involvement of G proteins was addressed, it could be demonstrated that opioid agonist stimulation of PDE activity was not mediated via the same G proteins as those that mediated opioid inhibition of adenylate cyclase activity. Previous treatment of NG108–15 cells with PTX almost completely abolished the ability of the opioid agonist to attenuate intracellular [^{3}H]cAMP production (Fig. 5). A parallel decrease in [^{32}P]ADP-ribosylation was observed with this paradigm of PTX pretreatment (Fig. 4). However, in the absence of PDE inhibitors the opioid agonist decreased the intracellular cAMP level significantly (Fig. 6). Thus, this decrease in the intracellular cAMP level in PTX-treated hybrid cells must reflect opioid agonist stimulation of PDE activity. Furthermore, this opioid receptor function was not mediated by G proteins that are PTX substrates. It is possible that opioid agonist stimulation of PDE activity was mediated without involvement of any G protein, but most likely the opioid

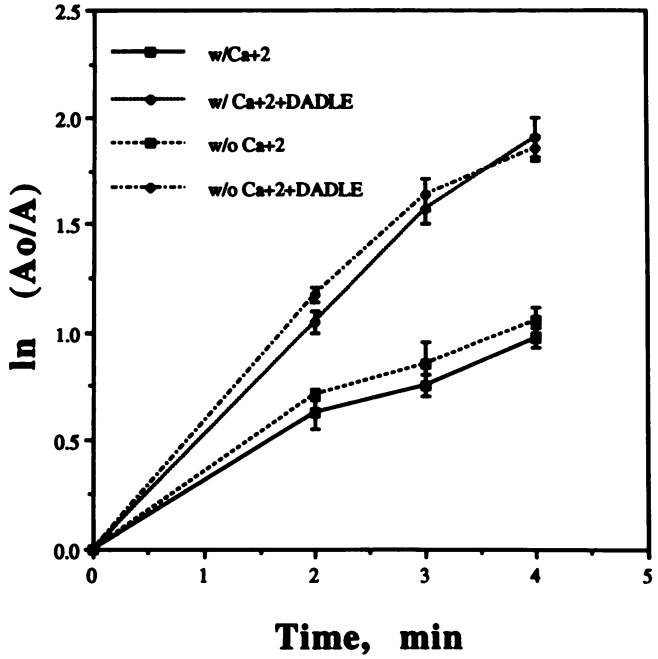

Fig. 12. Effect of extracellular Ca^{2+} on [^{3}H]cAMP degradation. NG108–15 cells were labeled with [^{3}H]adenine and the [^{3}H]cAMP level was elevated with 5 μM PGE_1 as described in Materials and Methods. The cells were then washed with KRHB containing 1 mM EGTA and no Ca^{2+} for the experiments in which extracellular Ca^{2+} was removed. The rate of [^{3}H]cAMP degradation was then measured in the absence (□) or in the presence (♦) of 100 nM DADLE in normal KRHB or in the absence (■) or in the presence (◇) of 100 nM DADLE in KRHB containing no Ca^{2+}.

stimulation of PDE activity was due to interaction of the δ-opioid receptor with G proteins that are not PTX substrates. The ability of the δ-opioid receptor to interact with multiple PTX substrates simultaneously has been demonstrated recently. Roerig *et al.* (8), using the properties of cholera toxin, demonstrated that in NG108–15 cells δ-opioid receptors could interact with $G_{i2\alpha}$, $G_{i3\alpha}$, and $G_{o\alpha}$ with the same affinity. Therefore, it is likely that the δ-opioid receptor interacted with a G protein that is not a PTX substrate, to elicit the stimulation of PDE.

The insensitivity of DADLE stimulation of PDE activity to PTX treatment could be due to the presence of multiple populations of opioid receptors in the NG108–15 cells. Whereas one population of receptors is involved in inhibition of adenylate cyclase activity, another might be involved in stimulation of the PDE activity. But from the dose-dependent studies using different agonists it is seen that the apparent population of receptors in NG108–15 cells that regulate PDE activity is the same as the population of receptors that regulate adenylate cyclase activity, i.e., δ-opioid receptors. Furthermore, chronic opioid agonist treatment produced parallel desensitization in both the opioid agonist-mediated stimulation of PDE activity and the opioid inhibition of adenylate cyclase activity (Fig. 10). Therefore, it is likely that the observed stimulation of PDE activity by the opioid agonist is due to activation of the same population of opioid receptors as those that mediate the inhibition of adenylate cyclase activity.

It is of interest that after chronic treatment the rate of cAMP degradation in the desensitized NG108–15 cells was lower than that in control cells, when the assays were carried out in the presence of the opioid antagonist naloxone (Fig. 10). Similarly to those of the δ-opioid receptors, desensitization and down-regulation of receptors were observed when the muscarinic receptors in NG108–15 cells were chronically activated (24). In addition, receptor down-regulation and desensitization were accompanied by an increase in intracellular cAMP levels if the agonist was removed from the receptor either by washing or by competition with antagonist. In the case of muscarinic receptors, this increase in the intracellular cAMP level could be explained partly by a decrease in the cAMP degradation rate (12). In the current studies, we demonstrated that after chronic DADLE treatment a parallel decrease in PDE activity was observed. It appears that there is a common adaptation pathway in NG108–15 cells that is triggered by the activation of receptors that regulate intracellular cAMP levels in an inhibitory manner.

In earlier studies it was reported that opioid agonists can induce a translocation of calmodulin to the plasma membrane (13), possibly due to elevation of intracellular free Ca^{2+} levels. Recent studies with a Ca^{2+}-sensitive fluorescent dye demonstrated an increase in intracellular free Ca^{2+} levels upon addition of Leu-enkephalin or DADLE to NG108–15 cells (18, 19). Calmodulin can modulate the activities of several enzymes, including Ca^{2+}/calmodulin-dependent PDE (type I). Drugs such as the phenothiazines (25), W-7 (26), and fenoctimine (27), which are 'calmodulin antagonists,' block calmodulin binding to PDE type I, inhibiting formation of the holoenzyme structure and thus preventing Ca^{2+} activation of the PDE activity. When DADLE-stimulated PDE activity was studied with various PDE inhibitors, we observed that only W-7 could attenuate the opioid agonist effect. Other inhibitors, such as rolipram, are very potent in inhibiting the overall PDE activity but had little or no effect on opioid stimulation of PDE activity. Thus, it is likely that the opioid agonist is stimulating the type I PDE activity. Because the inhibitor used, W-7, could not distinguish the subtype of PDE type I, it is possible that the observed opioid effect was due to the PDE that uses cGMP as substrate. Nevertheless, a possible sequence of cellular events that lead to subsequent PDE type I activation after activation of the δ-opioid receptor can be formulated. Activation of the δ-opioid receptor by agonist results in GTP/GDP exchange on a G protein that is not a PTX substrate. Activation of this G protein elevates intracellular Ca^{2+} levels. Possible candidates for control of cellular Ca^{2+} levels are the inositol phosphate metabolites. The ability of a non-PTX substrate, G_q, to regulate phospholipase C has been reported (28). Although G_q has not been reported in NG108–15 cells and the ability of agonist to stimulate inositol phosphate metabolism has not been reported, it is possible that G proteins other than G_q and inositol phosphate metabolites other than inositol trisphosphate could be involved in cellular Ca^{2+} regulation. Elevation of the Ca^{2+} level could lead to translocation of Ca^{2+}/calmodulin complexes to the membrane, which has been reported (13), and subsequent activation of the membrane-bound PDE activity; the elevated level of Ca^{2+}/calmodulin complex could also simply activate the cytosolic PDE activity.

Whether the sequence of cellular events described above actually occurs remains to be demonstrated. Furthermore, the probable presence of adenylate cyclase type I, which is activated by Ca^{2+}/calmodulin (29), in NG108–15 hybrid cells could influence the effect of W-7 on PDE activity. Thus, to demonstrate the validity of the aforementioned scheme several lines of experimental evidence are needed, including coupling between the δ-opioid receptor and a G protein that is not a PTX substrate and the ability of an opioid agonist to stimulate inositol phosphate metabolism. Studies are being carried out currently to establish these observations. Also, whether this opioid agonist effect is limited to NG108–15 cells remains to be demonstrated. It is of interest that opioid binding sites were reported in one of the parent cell lines of NG108–15, neuroblastoma N18TG2 (30), but the clonal cell line does not express Ca^{2+}/calmodulin-dependent PDE activity (31). In the other parent cell line, rat glioma C6BU1, Ca^{2+}/calmodulin-dependent PDE is expressed (32) but opioid binding sites are absent (33). Nevertheless, current studies suggest that intracellular cAMP levels in the NG108–15 cells can be regulated by multiple pathways. The agonist can inhibit adenylate cyclase activity and stimulate PDE activity. Just by monitoring the level of one second messenger, cAMP, we can see that the final observed level is the product of multiple cellular events. Therefore, the final cellular response to the activation of the δ-opioid receptor must be the product of the activation of multiple cellular effector systems. This hypothesis has been supported partially by our recent findings of δ-opioid receptor coupling with multiple G proteins (8).

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