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Inverse Agonistic Effect of ICI-174,864 on the Cloned δ-Opioid Receptor: Role of G Protein and Adenylyl Cyclase Activation

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SUMMARY

Previous studies have established that the δ-selective antagonist ICI-174,864 exhibits negative intrinsic activity at the δ-opioid receptors in NG108-15 membranes. To determine whether ICI-174,864 can function as a true inverse agonist in intact cells, its ability to stimulate cAMP accumulation was examined in a human embryonic kidney 293 cell line (293/DOR) expressing the cloned murine δ-opioid receptor. Forskolin-stimulated cAMP accumulation in the 293/DOR cells was dose-dependently suppressed by the δ-selective agonist [D-Pen^2, D-Pen^5]enkephalin, and such inhibition was abolished by pertussis toxin or the opiate antagonist naloxone. In contrast, ICI-174,864 significantly potentiated the forskolin response. The ICI-174,864-induced enhancement of the forskolin response exhibited dose-dependency and was antagonized by [D-Pen^2,D-Pen^5]enkephalin and blocked by pertussis toxin. Neither ICI-174,864 nor pertussis toxin elevated the basal level of cAMP accumulation in the absence of forskolin. Other opiate antagonists, such as naloxone and naltrindole, were ineffective in enhancing the forskolin-stimulated cAMP accumulation. Elevation of cAMP levels in response to the activation of G_s (through either ligand-bound receptor or point mutation on α_s) was also potentiated by ICI-174,864. Our results indicate that ICI-174,864 behaves as an inverse agonist in human embryonic kidney 293 cells stably expressing the δ-opioid receptor. The inverse agonistic effect of ICI-174,864 seemed to require G_i proteins and was clearly manifested when adenylyl cyclase was activated.

Receptors that use G proteins for signal transduction constitute a large number of biological detectors for hormones, neurotransmitters, photons, and odorants. The primary mode of activation for the superfamily of G protein-coupled receptors is via the binding of agonists, whereas antagonists block this productive interaction. Biochemical and pharmacological analyses of antagonists acting at various G protein-coupled receptors have revealed the presence of inverse agonists (also known as negative antagonists) that can produce effects opposite those of the corresponding agonists. To accommodate the actions of inverse agonists, a two-state model has been rejuvenated in which the receptor exists in equilibrium between the inactive conformation (R) and a spontaneously active conformation (R*) that can couple to G proteins in the absence of agonists (for a review, see Ref. 1). Inverse agonists acting at G protein-coupled receptors are thought to abolish spontaneous, agonist-independent activity by decreasing the propensity of the receptor to assume an active state (2). The mechanism for the induction of spontaneously active receptors is not clear. Point mutations (3, 4) are known to promote the formation of constitutively active G protein-coupled receptors, whereas overexpression (5; for a review, see Ref. 6) may facilitate the detection of these molecular entities by increasing the amount of constitutively active receptors.

Because opioid receptors can associate tightly with G_i/G_o proteins (7), they are prime candidates for study of the properties of inverse agonists. The δ-opioid receptor antagonist ICI-174,864 (*N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) has been shown to inhibit basal GTPase activity in membranes prepared from NG108-15 cells (8). Thermodynamic analysis of the binding isotherms of ICI-174,864 further indicated that it can act as an inverse agonist in NG108-15 cell membranes (9). More recently, it has been demonstrated that ICI-174,864 inhibits basal binding of [^{35}S]GTPγS to membranes of Rat 1 fibroblasts that express the cloned δ-opioid receptor (10). However, ICI-174,864 fails to elicit negative intrinsic activity in intact NG108-15 cells. Along with these findings came the issue of whether δ-opioid receptors can truly exist in an unliganded, active conformation in intact cells. Validation of inverse agonist activity *in vivo* was recently demonstrated in transgenic mice with myocardial overexpression of the β_2-adrenoceptor (11). In the current study, we investigated whether the δ-opioid receptor can adopt an unliganded, ac-

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ABBREVIATIONS: DPDPE, [D-Pen^2,D-Pen^5]enkephalin; PTX, pertussis toxin; HEK, human embryonic kidney; hCG, human choriogonadotropin; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

tive state in cells that normally do not express the receptor. If so, ICI-174,864 would be expected to stimulate cAMP formation on binding to the δ-opioid receptor. A major criterion for the receptor to achieve such a conformational state is the availability of suitable G proteins that can functionally interact with the receptor. HEK 293 cells have been shown to provide a suitable environment for δ-opioid receptor-mediated signaling (12). Thus, we examined the ability of ICI-174,864 to act as an inverse agonist in HEK 293 cells that stably express the δ-opioid receptor.

Materials and Methods

Reagents. The δ-opioid receptor (in the pCDM8 vector; Ref. 13) was kindly provided by Dr. Chris Evans (University of California, Los Angeles). PTX was purchased from List Biological Laboratories (Campbell, CA). hCG was supplied by the National Pituitary Agency (Bethesda, MD). HEK 293 cells were obtained from American Type Culture Collection (CRL-1573; Rockville, MD). [^{3}H]Adenine was purchased from Amersham (Arlington Heights, IL). [^{3}H]DPDPE (40.7 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Plasmid purification columns were obtained from Qiagen (Studio City, CA). Norbinaltorphimine and ICI-174,864 were purchased from Research Biochemicals International (Natick, MA). ICI-154,129 was obtained from Tocris Cookson (Bristol, UK). Cell culture reagents were supplied by GIBCO (Grand Island, NY), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Infection and transient transfection of HEK 293 cells. HEK 293 cells were maintained at 5% CO_2 at 37° in Earle's minimum essential medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cDNA encoding the δ-opioid receptor was excised from pCDM8 and subcloned into the retroviral expression vector pMV-7 at *Eco*RI sites. Then, 20 μg of pMV-7 with or without the δ-opioid receptor insert was transfected into retroviral packaging PA12 cells through standard calcium phosphate precipitation. Subsequent infection of HEK 293 cells with conditioned medium from G-418-selected PA12 cells was performed as previously described (14). For transient transfections, HEK 293 cells were seeded onto 12-well plates at 2×10^5 cells/well 1 day before transfection. The various cDNAs were purified via Qiagen columns and transfected using the DEAE-dextran method as previously described (15).

Receptor binding assays. Membranes from HEK 293 cells expressing the cloned murine δ-opioid receptor (HEK 293/DOR) were prepared according to published protocols (15). Cell membranes (30–50 μg) were incubated for 1 hr at 25° with 0.01–15 nM [^{3}H]DPDPE in a final volume of 500 μl of 50 mM Tris·HCl, pH 7.4. Bound ligand was separated by filtration through Whatman GF/B glass filters using a PHD cell harvester (Cambridge Technology, Watertown, MA). Radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of 10 μM DPDPE. K_d and B_{max} values were calculated by Scatchard analysis.

cAMP accumulation. Infected HEK 293 cells were seeded onto 24-well plates at 5×10^4 cells/well in 0.5 ml of growth medium. At 24 hr, cells were labeled with 0.5 ml of minimal essential medium containing 1 μCi of [^{3}H]adenine and 1% fetal calf serum in the absence or presence of PTX (100 ng/ml). Similar seeding and labeling conditions were used for NG108-15 cells, except that the cells were cultured in Dulbecco's modified Eagle's medium. In transient transfections, cells were labeled under similar conditions at 24 hr after transfection. At 16–24 hr after labeling, cells were assayed for cAMP accumulation as previously described (14). Results are expressed as the ratios of [^{3}H]cAMP to total [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]cAMP pools.

Results

Effects of ICI-174,864 on forskolin-stimulated cAMP accumulation. The stable HEK 293/DOR cell line was established by infecting HEK 293 cells with retroviral particles harboring the δ-opioid receptor cDNA and subsequent selection under G-418. The G-418-resistant HEK 293/DOR cells were used for further studies. Binding of the δ-selective agonist [^{3}H]DPDPE to membranes prepared from HEK 293/DOR cells yielded a K_d value of 8.50 ± 0.67 nM (three experiments) and a B_{max} value of 966 ± 46 fmol/mg of protein. Such binding characteristics of the cloned δ-opioid receptor in HEK 293/DOR cells were similar to those observed when the same receptor was expressed in Chinese hamster ovary cells (16). No specific [^{3}H]DPDPE binding was detectable in noninfected or vector-infected control cells. However, compared with the parental HEK 293 cells, HEK 293/DOR cells exhibit impaired responsiveness to forskolin (Fig. 1). Forskolin dose-dependently stimulated cAMP accumulation in HEK 293 cells with an EC_{50} of ~35 μM. Due to the poor solubility of forskolin, a complete dose-response curve for forskolin-induced stimulation of adenylyl cyclase activity in HEK 293/DOR cells could not be achieved. However, at concentrations of ≤500 μM, forskolin-induced cAMP accumulations in HEK 293/DOR cells were significantly less than those observed in HEK 293 cells (Fig. 1). The reduced ability of forskolin to stimulate adenylyl cyclase was not observed in HEK 293 cells infected with the pMV-7 vector alone (data not shown).

To determine the functionality of the δ-opioid receptor, we examined the ability of DPDPE to inhibit forskolin-induced cAMP accumulation in HEK 293/DOR cells. Forskolin (50 μM, a concentration close to the EC_{50} value of forskolin in HEK 293 cells) increased the cAMP accumulation by ~250-fold compared with basal adenylyl cyclase activity. The application of DPDPE to HEK 293/DOR cells led to a marked inhibition of forskolin-stimulated cAMP accumulation. The inhibitory effect of DPDPE was dose dependent and exhibited an EC_{50} value of ~0.1 nM with maximal inhibition of ~80% observed at 3 nM (Fig. 2). At 100 nM, neither μ-selective ([D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin) nor κ-selective (U50,488) agonists inhibited the forskolin-stimulated cAMP accumulation in HEK 293/DOR cells (data not shown). These

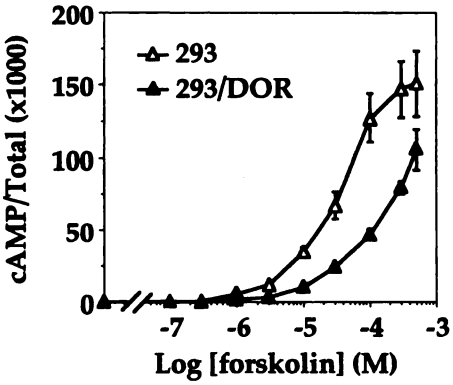

Fig. 1. Forskolin-stimulated cAMP accumulation in uninfected HEK 293 and HEK 293/DOR cells. Cells were labeled with [^{3}H]adenine (1 μCi/ml) and then assayed for cAMP accumulation in response to varying concentrations of forskolin. Data are the mean ± standard error of three independent experiments. At concentrations of ≥3 μM, the forskolin response in HEK 293/DOR cells was significantly lower than that observed in uninfected HEK 293 cells (paired *t* test, $p < 0.05$).

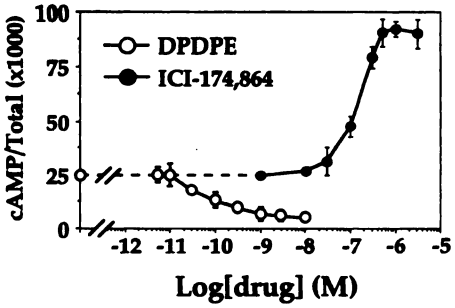

Fig. 2. Effects of DPDPE and ICI-174,864 on forskolin-stimulated cAMP accumulation in HEK 293/DOR cells. HEK 293/DOR cells were labeled with [^{3}H]adenine (1 μCi/ml) and then assayed for cAMP accumulation in response to forskolin (50 μM) in the absence or presence of varying concentrations of DPDPE or ICI-174,864. Data are the mean ± standard error of three independent experiments. Basal cAMP accumulation (in the absence of forskolin) was 0.23 ± 0.09. At a concentration of ≥0.03 nM DPDPE or ≥0.1 μM ICI-174,864, the forskolin response was significantly different from the control (paired *t* test, $p < 0.05$).

results are in agreement with previous studies on the signaling properties of the δ-opioid receptor in HEK 293 cells utilizing transient transfection assays (12).

In contrast, the δ-selective antagonist ICI-174,864 significantly enhanced the forskolin-stimulated cAMP accumulation in a dose-dependent manner (Fig. 2). ICI-174,864 potentiated the forskolin response maximally by 4-fold and showed an EC_{50} value of ~0.2 μM. In the absence of forskolin, ICI-174,864 was unable to stimulate the basal activity of adenylyl cyclase (Fig. 3). We next examined the effects of PTX on the ICI-174,864 response. Pretreatment of the cells with PTX for 16 hr before the cAMP accumulation assays abolished the ability of DPDPE and ICI-174,864 to inhibit and enhance, respectively, the forskolin response (Fig. 3). PTX alone did not affect the ability of forskolin to stimulate adenylyl cyclase. Sensitivity to PTX indicates that the potentiating effect of ICI-174,864 was mediated via the δ-receptor and required functional coupling to G_i/G_o-like proteins.

Selectivity of the negative intrinsic activity of ICI-174,864. We further examined whether the negative intrinsic activity of ICI-174,864 could be seen with other opiate

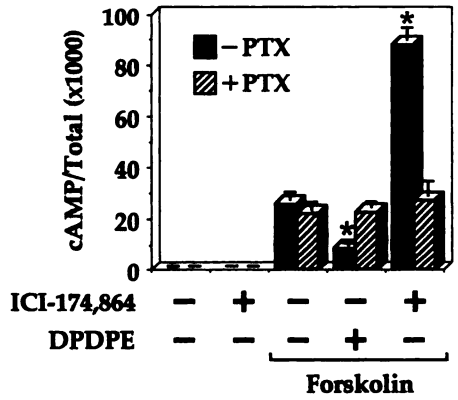

Fig. 3. PTX sensitivity of opioid ligands on the modulation of forskolin-stimulated cAMP accumulation in HEK 293/DOR cells. HEK 293/DOR cells were labeled with [^{3}H]adenine (1 μCi/ml) with or without PTX (100 ng/ml). Cells were assayed for cAMP accumulation in the absence or presence of forskolin (50 μM), DPDPE (10 nM), or ICI-174,864 (1 μM) as indicated. Data are the mean ± standard error of four independent experiments. *, cAMP accumulation was significantly different from that obtained in the presence of forskolin alone (paired *t* test, $p < 0.05$).

antagonists. Forskolin-stimulated cAMP accumulations in HEK 293/DOR cells were determined in the absence or presence of one of several opiate antagonists. At concentrations of 1 μM, only ICI-174,864 significantly potentiated the forskolin response (Table 1). Both naloxone (a nonselective opiate antagonist) and norbinaltorphimine (a κ-selective antagonist) were ineffective in enhancing the forskolin response. Other δ-selective antagonists such as *N*-benzylnaltrindole,[2] naltrindole, and ICI-154,129 (Table 1) were also unable to potentiate the forskolin response. These results indicate that the ability of an antagonist to act as an inverse agonist is intrinsic to the compound and is independent of its ability to bind to the receptor.

As an antagonist, ICI-174,864 should possess the ability to block responses induced by an agonist. By the same analogy, DPDPE should antagonize effects produced by ICI-174,864. Responses to forskolin in HEK 293/DOR cells were therefore determined in the presence of ICI-174,864 with or without an opioid agonist. As expected, potentiation of the forskolin response by ICI-174,864 was suppressed by DPDPE (Fig. 4), whereas both [D-Ala^2,*N*-MePhe^4,Gly^5-ol]enkephalin (μ-selective) and U50,488 (κ-selective) were unable to diminish the ICI-174,864 response.[3] In the presence of 1 μM ICI-174,864, DPDPE dose-dependently inhibited the forskolin-stimulated cAMP accumulation with an EC_{50} value of ~1 nM (Fig. 4). At a concentration of ~10 nM, DPDPE suppressed cAMP formation beyond the level that was normally stimulated with forskolin in the absence of ICI-174,864. Both naloxone and naltrindole effectively attenuated the capacity of ICI-174,864 to enhance the forskolin response (Table 1). The ICI-174,864 response thus seemed to be mediated through the δ-opioid receptor.

If ICI-174,864 were indeed acting as an inverse agonist, then the δ-opioid receptors must be able to assume a ligand-independent, active state that can constitutively inhibit adenylyl cyclase. Compared with the parental HEK 293 cells, the forskolin-stimulated cAMP accumulation in HEK 293/DOR cells was significantly reduced (Table 1 and Fig. 1). Furthermore, when assayed in the presence of ICI-174,864, the forskolin response in HEK 293/DOR cells became comparable to that of uninfected HEK 293 cells (Table 1). ICI-174,864 seemed to relieve the constitutive suppression of adenylyl cyclase so that a maximal forskolin response can be achieved.

We next tested whether ICI-174,864 can potentiate G_s-stimulated cAMP accumulation. The HEK 293 cells were transiently cotransfected with cDNAs encoding the rat luteinizing-hormone receptor and the δ-opioid receptor. The transfected cells were assayed for cAMP accumulation in the presence of 10 ng/ml hCG with or without opioid ligands. As shown in Fig. 5, the hCG response was inhibited by 10 nM DPDPE but enhanced by 1 μM ICI-174,864. The hCG response was doubled in the presence of ICI-174,864 (Fig. 5). Like the forskolin response in HEK 293/DOR cells, the hCG response in the transfected cells was unaffected by naloxone (1 μM), whereas DPDPE (10 nM) blocked the potentiating effect of ICI-174,864. Likewise, in HEK 293 cells coexpressing the δ-opioid receptor and a constitutively activated mutant α_s (α_s-Q227L; Ref. 17), ICI-174,864 significantly stimu-

[2] H. Beck and Y. H. Wong, unpublished observations.
[3] T. T. Chiu and Y. H. Wong, unpublished observations.

TABLE 1

Effects of opioid ligands on forskolin-stimulated cAMP accumulation in HEK 293/DOR and uninfected HEK 293 cells

HEK 293/DOR and noninfected HEK 293 cells were assayed for cAMP accumulation (37° for 30 min) in the presence of 50 μM forskolin with or without the indicated ligands. Each antagonist was used at 1 μM except when added together with ICI-174,864, 10 μM naloxone or naltrindole was used. Data are the mean ± standard error of three independent experiments.

Cell line	Ligands in addition to 50 μM forskolin	cAMP accumulation	
		[^{3}H]cAMP/total[a]	% Forskolin response[b]
HEK 293/DOR	None	29.8 ± 3.9	100
	Naloxone	30.7 ± 2.8	103
	Norbinaltorphimine	29.7 ± 5.2	99
	Naltrindole	27.9 ± 3.3	94
	ICI-154,129	32.4 ± 3.1	109
	ICI-174,864	96.3 ± 2.9	323
	ICI-174,864 and naloxone	30.4 ± 2.1	102
	ICI-174,864 and naltrindole	25.8 ± 4.4	87
HEK 293	None	90.6 ± 5.5	304
	ICI-174,864	82.4 ± 6.1	277

[a] Results are expressed as the ratio of [^{3}H]cAMP to total (×1000). Basal values ranged from 0.11 ± 0.09 to 0.45 ± 0.21.
[b] Adenylyl cyclase activities are expressed as percentage of the forskolin-stimulated response as measured in the absence of opioid ligands in HEK 293/DOR cells.

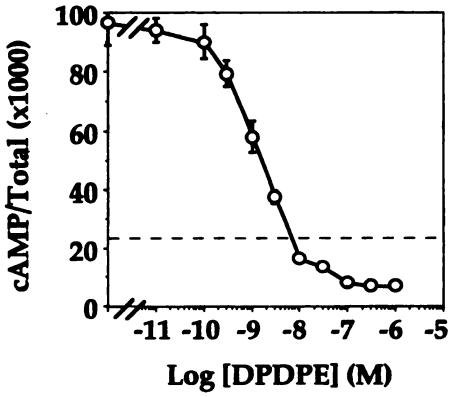

Fig. 4. DPDPE-induced inhibition of forskolin-stimulated cAMP accumulation in the presence of ICI-174,864. HEK 293/DOR cells were labeled with [^{3}H]adenine (1 μCi/ml) and then assayed for cAMP accumulation in response to forskolin (50 μM) in the presence of ICI-174,864 (1 μM) and varying concentrations of DPDPE (0.01 nM to 1 μM). *Dashed line*, forskolin-stimulated activity as measured in the absence of ICI-174,864. Data are the mean ± standard error of three independent experiments. Basal cAMP accumulation was 0.17 ± 0.08. At a concentration of ≥0.3 nM DPDPE, the forskolin response was significantly inhibited (paired *t* test, $p < 0.05$).

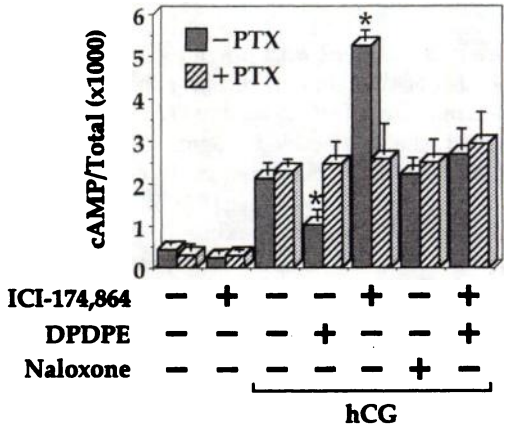

Fig. 5. Inverse agonistic effect of ICI-174,864 in transiently transfected HEK 293 cells. HEK 293 cells were transiently transfected (DEAE-Dextran method) with cDNAs encoding the luteinizing-hormone receptor (in pCIS, 0.15 μg/ml) and δ-opioid receptor (in pCDM8, 0.25 μg/ml). Transfected cells were labeled with [^{3}H] adenine (1 μCi/ml) in the absence or presence of PTX (100 ng/ml) and subsequently assayed for responses to hCG (10 ng/ml) with or without opioid ligands. Where indicated, 10 nM DPDPE, 1 μM naloxone, or 1 μM ICI-174,864 was used. Data are the mean ± standard error of three independent experiments. *, cAMP accumulation was significantly different from that obtained in the presence of hCG alone (paired *t* test, $p < 0.05$).

lated the basal cAMP accumulation (Fig. 6). The potentiating effects of ICI-174,864 on both hCG- and α_s-Q227L-stimulated cAMP accumulations were abolished by PTX treatment (Figs. 5 and 6). However, PTX alone did not affect the hCG- or α_s-Q227L-stimulated adenylyl cyclase activities. These findings indicate that ICI-174,864 can enhance G_s- as well as forskolin-stimulated cAMP accumulation.

Having demonstrated the inverse agonistic effect of ICI-174,864 in intact HEK 293/DOR cells, we then examined the whole-cell response of NG108-15 to ICI-174,864. No inverse agonist activity was observed for 1 μM ICI-174,864 in intact NG108-15 cells, even when adenylyl cyclase was concurrently stimulated by forskolin (Fig. 7) or prostaglandin E_1 (data not shown). Under similar assay conditions, DPDPE (100 nM) inhibited the forskolin response by 50%, whereas ≤5 μM ICI-174,864 did not elicit an enhancement effect.[4] This is in direct contrast to HEK 293/DOR cells, in which 1 μM ICI-174,864 enhanced the forskolin-induced cAMP accumulation over a range of forskolin concentrations (0.1–500 μM).

[4] L. Y. Yung, J. S. C. Chan, and Y. H. Wong, unpublished observations.

It seemed that the ability of ICI-174,864 to act as an inverse agonist in intact cells may be cell type specific.

Discussion

Previous studies have implicated ICI-174,864 as an inverse agonist at the δ-opioid receptor because it inhibits GTPase activity (8, 10) and exhibits thermodynamic properties typically seen with inverse agonists (9). However, these studies were performed on cell membranes, and ICI-174,864-induced enhancement of basal adenylyl cyclase activity could not be demonstrated in intact cells. It was postulated that the formation of unliganded constitutively active receptors was prohibited in intact cells, perhaps by factors found in the cytosol. By using an HEK 293 cell line expressing the δ-opioid receptor, we have successfully shown that ICI-174,864 can indeed act as an inverse agonist in intact cells. This notion is supported by several observations: (i) ICI-174,864 dose-dependently enhanced the forskolin-stimulated adenylyl cyclase

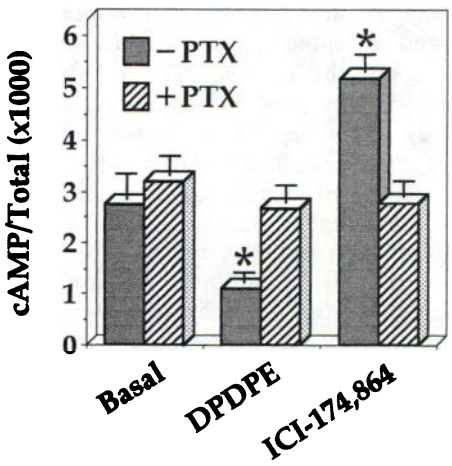

Fig. 6. Potentiation of α_s-stimulated cAMP accumulation by ICI-174,864 in transiently transfected HEK 293 cells. HEK 293 cells were transiently transfected with cDNAs encoding α_s-Q227L (in pcDNAI, 0.25 μg/ml) and δ-opioid receptor (0.25 μg/ml). Transfected cells were labeled with [^{3}H]adenine (1 μCi/ml) in the absence or presence of PTX (100 ng/ml) and subsequently assayed for responses to either 100 nM DPDPE or 1 μM ICI-174,864. Data are the mean ± standard error of four independent experiments. *, cAMP accumulation was significantly different from that obtained in the absence of opioid ligands (paired *t* test, $p < 0.05$).

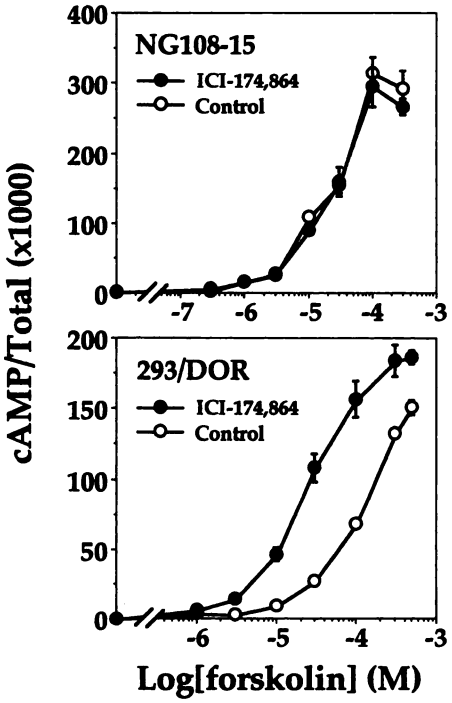

Fig. 7. Effect of ICI-174,864 on forskolin-stimulated cAMP accumulation in intact NG108-15 and HEK 293/DOR cells. NG108-15 and HEK 293/DOR cells were labeled with [^{3}H]adenine (1 μCi/ml) and then assayed for cAMP accumulation in response to varying concentrations of forskolin with or without 1 μM ICI-174,864. Data are the mean ± standard error of three independent experiments. Basal values for NG108-15 and HEK 293/DOR cells were 1.74 ± 0.7 and 0.21 ± 0.06, respectively. At concentrations of ≥3 μM, the forskolin response in HEK 293/DOR cells was significantly enhanced by ICI-174,864 (paired *t* test, $p < 0.05$).

activity in HEK 293/DOR cells; (ii) enhancement of the forskolin response by ICI-174,864 was blocked in the presence of DPDPE or naloxone; (iii) PTX potently abolished the ICI-174,864 response, presumably by preventing the interaction of G_i proteins with the unliganded, spontaneously active opioid receptors; and (iv) the magnitude of ICI-174,864-enhanced forskolin response in HEK 293/DOR cells was comparable to that measured with forskolin alone in uninfected HEK 293 cells, strongly suggesting that a significant amount of δ-opioid receptors are spontaneously active in the HEK 293/DOR cells, resulting in constitutive suppression of the forskolin-stimulated adenylyl cyclase activity. Some of these observations were extended to HEK 293 cells transiently coexpressing the δ-opioid receptor.

Compelling evidence is available to indicate that the level of receptor expression can influence the specificity of receptor/G protein coupling (for a review, see Ref. 6) and presumably the conformational state of the receptor. By analogy to the overexpression of the β-adrenoceptor (5), overexpression of the δ-opioid receptors may increase the amount of R* and thus suppress the basal adenylyl cyclase activity. Inverse agonists should therefore elevate the basal activity of adenylyl cyclase by shifting the δ receptor equilibrium to the inactive R state. However, we were unable to detect a potentiating effect of ICI-174,864 on the basal cAMP accumulation. Enhancement of adenylyl cyclase activity by ICI-174,864 could be observed only with forskolin- or G_s-stimulated activity. Inhibition of basal adenylyl cyclase activity has always been less prominent and more difficult to detect. Because α_i and α_s seem to interact with adenylyl cyclase at distinct sites (18), it is conceivable that GTP-bound α_i subunits may preferentially recognize the active conformation of adenylyl cyclase. In such a case, even when the δ-opioid receptor adopts a ligand-independent and active conformation, activation of the corresponding G_i proteins might have minimal effect on the basal activity of adenylyl cyclase. Circumstantial evidence in support of this hypothesis comes from the observation that constitutively active mutant α_{i2} polypeptides do not inhibit basal cAMP accumulation in NIH-3T3 cells but potently suppress the forskolin-stimulated activity (15). This may also explain why there was an apparent lack of stimulation of the basal adenylyl cyclase activity by ICI-174,864 in intact NG108-15 cells (9). Because concurrent stimulation of adenylyl cyclase did not unmask inverse agonist activity in intact NG108-15 cells, the possible involvement of cytosolic factors that prohibit the formation of R* cannot be eliminated. Perhaps the composition and stoichiometry of signal transduction molecules within each cell type influence the propensity for the formation of R*.

An interesting but puzzling finding was that although PTX completely abolished DPDPE-induced inhibition of cAMP accumulation, it did not enhance the forskolin response. If one assumes that the δ-opioid receptors can spontaneously adopt a ligand-independent active conformation that can constitutively inhibit forskolin-stimulated adenylyl cyclase activity, activation of G_i must be involved. Then, by disrupting the receptor/G_i interaction, PTX treatment should allow the forskolin response to approach the "normal" uninhibited level (compare forskolin responses in HEK 293/DOR and uninfected HEK 293 cells, Table 1). However, PTX did not alter the forskolin response in HEK 293/DOR cells. A plausible explanation is that the fraction of G_i proteins associated with the unliganded, spontaneously active receptors (R*G complex) is less accessible to PTX. It has been shown that the undissociated GDP-bound form of G_i/G_o proteins is preferred

by PTX (19). The spontaneously active δ-opioid receptors might lead to the formation of GTP-bound α_i subunits, which are poor substrates for PTX. It should also be noted that the putative receptor interacting domain and the PTX-catalyzed ADP-ribosylation site (Cys351) are both located at the extreme carboxyl terminus of the α subunit of G_i; it is likely that the receptor may reciprocally prevent PTX from interacting with G proteins.

It is generally assumed that the inhibition of spontaneous receptor activity requires the destabilization of receptor/G protein complexes and that inverse agonists promote receptor/G protein dissociation. In accordance with the two-state model, a ligand that destabilizes receptor/G protein coupling, one that selectively binds to the inactive form of the receptor, or both will act as an inverse agonist. The current report introduces another aspect by showing that PTX can effectively abolish the ability of ICI-174,864 to enhance the forskolin response. Because the forskolin response in PTX-treated cells remained constitutively suppressed (i.e., the same as that of the untreated HEK 293/DOR cells but much lower than those of uninfected HEK 293 cells), the loss of the inverse agonistic effect of ICI-174,864 could not be explained by the decreased formation of spontaneously active receptors. With the assumption that PTX does not readily ADP-ribosylate G_i/G_o proteins in R*G complexes (as discussed above), other effects of PTX should be contemplated. PTX binds to cell surface glycoproteins such as haptoglobin, fetuin, and the ganglioside G_{D1a} via its B oligomer, which is devoid of ADP-ribosyltransferase activity. The B oligomer of PTX has been shown to activate platelets (20), inhibit growth cone guidance in neurons (21), and mobilize intracellular Ca^{2+} in human T lymphocytes (22). Perhaps the PTX sensitivity of ICI-174,864 is independent of the ADP-ribosyltransferase activity of the toxin. G protein-independent effects of PTX can be readily tested with the use of the B oligomer instead of the holotoxin.

The PTX sensitivity of the inverse agonistic property of ICI-174,864 suggests that the ligand preferentially binds to G protein-coupled δ receptors or that it binds to uncoupled receptors but requires subsequent coupling to G proteins. Both scenarios are inconsistent with the two-state model for receptor activation. The two-state model does not embody the role of G proteins in the induction and/or stabilization of the different conformational states of the receptor; only the active receptor R* has the ability to activate G proteins. Based on the original ternary complex model described by De Lean *et al.* (23), a modified version was proposed to accommodate the existence of a constitutively activated mutant β_2-adrenergic receptor (4). In this modified version, the receptor can isomerize to an active state, which can then interact with G proteins by forming the R*G complex. The G protein dependency of the ICI-174,864 effect implies that a similar complex (RG) may exist for the inactive receptor. Unlike R*G, RG represents a precoupled state that does not regulate downstream effector molecules. The binding of ICI-174,864 to the RG complex is expected to reduce the formation of R*G complexes, whereas PTX treatment will prevent the generation of RG and induce the accumulation of uncoupled R by ADP-ribosylating the G protein in RG.

The concept of a precoupled receptor/G protein state has endured intensive scrutiny. Substantial biochemical evidence has been accumulated in support of the notion that some receptors are precoupled to G proteins in the absence of ligands. Many G_i-coupled receptors, including the α_2-adrenergic (24) and all three types of opioid receptors (7, 25), seemed to fall into this category. The precoupled receptor/G protein complex may exist in an inactive state (RG) or a spontaneously active form (R*G). Although our results suggest that the inverse agonistic activity of ICI-174,864 may be transduced via the RG complex, it remains possible for the R*G or R to have higher affinities for the negative antagonist. In G_q-linked receptor systems, inverse agonists may bind preferentially to the uncoupled receptor. The binding of inverse agonists to the 5-hydroxytryptamine$_{2C}$ receptor is promoted by GTPγS, which presumably shifts the equilibrium of 5-hydroxytryptamine$_{2C}$ receptors to the uncoupled state (26, 27). Similarly, inverse agonists promote an inactive conformation of the G_s-coupled β_2-adrenoceptor (2). In light of our current findings, the influence of PTX and GTPγS on the binding of ICI-174,864 to δ-opioid receptors should be examined in detail. Likewise, the resistance of R*G to PTX-catalyzed ADP-ribosylation should be assessed by *in vitro* assays using purified components. Such information will be invaluable in deciphering the mechanisms of action of inverse agonists.

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