0026-895X/01/6005-1076–1082$3.00
MOLECULAR PHARMACOLOGY
Vol. 60, No. 5
1017/940422
Mol Pharmacol 60:1076–1082, 2001
Printed in U.S.A.

Expression of α-Transducin in Chinese Hamster Ovary Cells Stably Transfected with the Human δ-Opioid Receptor Attenuates Chronic Opioid Agonist-Induced Adenylyl Cyclase Superactivation

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Received April 9, 2001; accepted August 13, 2001

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

ABSTRACT

To investigate the role of G-protein βγ subunits in δ-opioid signal transduction, we have transfected Chinese hamster ovary (CHO) cells stably expressing the human δ-opioid receptor (hDOR/CHO cells) with the G_{α}-subunit of transducin-1 (hDOR/T1/CHO). Inhibition of forskolin-stimulated adenylyl cyclase and phospholipase Cβ (PLCβ) activation was measured in each of these cell lines. Because PLCβ_3 activation in CHO cells has been shown to be mediated by free $G_{\beta\gamma}$ subunits derived from $G_{\alpha i/o}$, the action of transducin was confirmed by measuring a significant attenuation of (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80)-mediated maximal inositol-1,4,5-trisphosphate formation in transducin-expressing cells of 59 ± 12% compared with control cells. The acute inhibition of cAMP formation was unchanged between control and transducin-expressing cells. We show that cells stably expressing the human δ-opioid receptor exhibited a pertussis toxin-sensitive cAMP overshoot in response to chronic application of SNC80. After 4 h of pretreatment and washout with 100 nM SNC80, maximal forskolin-stimulated cAMP formation in hDOR/CHO cells increased by 229 ± 37% compared with buffer-treated cells. Expression of transducin in hDOR/CHO cells diminished this response: hDOR/T1/CHO cells showed no significant change in maximal forskolin-stimulated cAMP formation after pretreatment and washout. These data indicate that the expression of α-transducin scavenges free $G_{\beta\gamma}$ subunits and, furthermore, that free $G_{\beta\gamma}$ subunits play a role in opioid-mediated PLCβ activation and adenylyl cyclase superactivation, but not acute inhibition of forskolin-stimulated cAMP formation in hDOR/CHO cells.

Of the three distinct opioid receptors that have been identified (μ, κ, and δ), the δ-opioid receptor seems to have the most promise for mediating analgesia while minimizing such adverse side effects as addiction, respiratory depression, and constipation (Rapaka and Porreca, 1991; Quock et al., 1999). In 1994, the human δ-opioid receptor (hDOR) was cloned (Knapp et al., 1994; Simonin et al., 1994) and subsequently stably transfected into CHO cells for characterization (Malatynska et al., 1995). We have shown that the hDOR in this cell line exhibits a typical inhibition of forskolin-stimulated cAMP accumulation and a cAMP overshoot after chronic opioid agonist pretreatment. In this regard, Chinese hamster ovary cells stably expressing the human δ-opioid receptor (hDOR/CHO) have proven to be useful for studying adenylyl cyclase (AC) superactivation by δ-opioid-selective agonists (Malatynska et al., 1996).

The adenylyl cyclase superactivation phenomenon was first noted by Sharma et al. (1975). In their studies, NG108–15 cells, which express primarily mouse δ-opioid receptors, exhibited an inhibition of PGE_1-stimulated cAMP formation in response to acute treatment with morphine, whereas chronic morphine treatment followed by removal resulted in an increased PGE_1-stimulated cAMP accumulation. This opioid-mediated cAMP overshoot has since been demonstrated in a variety of systems as reviewed by Nestler (1993).

Although the original finding took place more than 25 years ago, the molecular mechanism of adenylyl cyclase superactivation has yet to be entirely elucidated. What has

This work was supported in part by a grant from the Arizona Disease Control Research Commission and a training grant from the National Institute on Drug Abuse.

ABBREVIATIONS: hDOR, human δ-opioid receptor; CHO, Chinese hamster ovary; AC, adenylyl cyclase; PLCβ, phospholipase Cβ; IP_1, inositol-1,4,5-trisphosphate; SNC80, (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; hDOR/CHO, Chinese hamster ovary cells stably expressing the human δ-opioid receptor; α-transducin, G_{α}-protein subunit of transducin-1; hDOR/T1/CHO, Chinese hamster ovary cells stably expressing both the human δ-opioid receptor and α-transducin; IMDM, Iscove's modified Dulbecco's medium.

been shown is the involvement of specific adenylyl cyclase isoforms, G-protein α and $\beta\gamma$ subunits, and kinases, depending on the system being studied (Avidor-Reiss et al., 1995; Ammer and Schulz, 1998; Chakrabarti et al., 1998a,b). In fact, the molecular mechanism of AC superactivation remains elusive and seems to be somewhat tissue-dependent: Ammer and Schulz (1998) have demonstrated the requirement for activated $G_{\alpha s}$ to show a cAMP overshoot in opioid-withdrawn NG108–15 cells (Ammer and Schulz, 1998). Chakrabarti et al. (1998a) have shown that in guinea pig ileum longitudinal muscle myenteric plexus preparations, chronic in vivo pretreatment with morphine results in a type of cAMP overshoot wherein a reversal from inhibition to stimulation of AC is observed (Chakrabarti et al., 1998a), and that activation of protein kinase C is required for ACII phosphorylation by the same pretreatment (Chakrabarti et al., 1998b). Furthermore, they were able to demonstrate an up-regulation of ACII (Chakrabarti et al., 1998b), which is an AC that has been shown to be conditionally stimulated by $G_{\beta\gamma}$ and $G_{\alpha s}$ (Sunahara et al., 1996). It is likely that, in this system, these events contribute to the cAMP overshoot caused by chronic morphine. In contrast, a CHO cell line stably transfected with the rat μ-opioid receptor exhibits AC superactivation that is unaffected by cycloheximide pretreatment, indicating that no up-regulation of AC or $G_{\alpha s}$ occurs in this system (Avidor-Reiss et al., 1995). These results indicate that although the cAMP overshoot phenomenon first demonstrated by Sharma et al. (1975) is common, the series of events leading up to this response can be variable. Some aspects of the cAMP overshoot, however, seem to be consistent, such as signaling through $G_{\beta\gamma}$.

Chronic opioid agonist-mediated adenylyl cyclase superactivation and the resultant cAMP overshoot have long been considered in vitro models for studying opioid tolerance and withdrawal (Sharma et al., 1975, 1977; Nestler, 1993; Malatynska et al., 1996; Ammer and Schulz, 1998). Although opioid-activated $G_{i/o}$-protein α subunits acutely inhibit cAMP formation, it has been hypothesized that the liberated $G_{\beta\gamma}$ subunits play a role in the compensatory increase in forskolin-stimulated cAMP formation. One method for examining this hypothesis is to interrupt signaling through $G_{\beta\gamma}$.

It has been shown that pertussis toxin-sensitive $G_{i/o}$-coupled receptors can activate phospholipase Cβ_2 (PLCβ_2) and PLCβ_3 via liberated $G_{\beta\gamma}$ subunits in various cell lines including CHO cells, and that CHO cells express PLCβ_3 exclusively (Camps et al., 1992; Dickenson and Hill, 1998). Here, we are demonstrating a pertussis toxin-sensitive SNC80-mediated increase in IP_1 formation in hDOR/CHO cells, indicating that δ-opioid receptor activation leads to PLCβ stimulation through $G_{\beta\gamma}$. Therefore, one would expect the SNC80-mediated increase in IP_1 formation in hDOR/CHO cells to be attenuated by interfering with $G_{\beta\gamma}$ signaling (Freund et al., 1994; Murthy and Makhlouf, 1996; Zhang et al., 1996).

A variety of in vitro methods has been employed to investigate the effects of signal transduction through $G_{\beta\gamma}$ subunits, including antisense knockdown and scavenge of free $G_{\beta\gamma}$. Because of its established role in $G_{\beta\gamma}$ scavenging by expression in a variety of systems (Federman et al., 1992; Avidor-Reiss et al., 1996; Yoshimura et al., 1996; Selbie et al., 1997; Olianas and Onali, 1999), we chose to use the cloned G-protein α subunit of retinal transducin-1 (α-transducin) to scavenge free $G_{\beta\gamma}$ subunits. We are demonstrating the importance of free $G_{\beta\gamma}$ subunits in mediating PLCβ_3 activation and adenylyl cyclase superactivation by stably transfecting α-transducin, into CHO cells stably expressing the human δ-opioid receptor (thereby creating hDOR/T1/CHO cells). It was our hypothesis that because both PLCβ_3 activation and adenylyl cyclase superactivation are dependent on $G_{\beta\gamma}$, the stable expression of α-transducin in hDOR/CHO cells will result in a decreased maximal SNC80-mediated IP_1 formation and a loss of the cAMP overshoot after chronic opioid agonist pretreatment.

Materials and Methods

Preparation of hDOR/T1/CHO Cells and Cell Culture. hDOR/CHO (Malatynska et al., 1995) were transfected with a pertussis toxin-insensitive mutant of the α-subunit of transducin-1 (hDOR/T1/CHO) as described previously (Varga et al., 2000). Both hDOR/CHO and hDOR/T1/CHO cells were plated in 24-well polystyrene plates (Costar, Cambridge, MA) at a density of 65,000 cells per well and grown in a humidified incubator at 37°C (5% CO_2) for 48 h in Ham's F-12 medium (Invitrogen, Carlsbad, CA) with 10% fetal calf serum before assay.

Forskolin-Stimulated cAMP Formation. This was done following a method modified from Gilman (1970). The growth medium was aspirated and replaced with serum-free Iscove's modified Dulbecco's medium (IMDM) (Invitrogen). In experiments involving opioid agonist pretreatment, either IMDM or 100 nM SNC80 (Tocris, Ballwin, MO) in IMDM was added to cells for 4 h. After this chronic treatment, the cells were washed three times with fresh IMDM, 15 min per wash. The IMDM was then aspirated and replaced with 5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma Chemical, St. Louis, MO) in IMDM. Adenylyl cyclase was stimulated with water-soluble forskolin [7-deacetyl-7-(*O*-N-methylpiperazino)-γ-butyryl, diHCl; Calbiochem, San Diego, CA]. Doses of SNC80 with 100 μM forskolin (agonist dose-response curve) or doses of forskolin alone (forskolin dose-response curve) were added to the wells, which were then placed in a humidified incubator at 37°C (5% CO_2) for 20 min. The forskolin was then aspirated and replaced with 150 μl of ice-cold Tris/EDTA buffer (50 mM Tris HCl, 4 mM EDTA, pH 7.5) to terminate the reaction. Each well was scraped to dislodge the cells, and the contents of each well were transferred to Microfuge tubes (Beckman Coulter, Fullerton, CA) and boiled for 10 min to lyse the cells. Lysate was centrifuged and 50 μl of supernatant was incubated with 50 μl of [^{3}H]cAMP (final concentration, 4 nM; PerkinElmer Life Sciences, Boston, MA) and 100 μl of protein kinase A (final concentration, 30 μg/ml; Sigma Chemical, St. Louis, MO) as a cAMP binding protein. cAMP standards were run in parallel. After a 2-h incubation at 4°C, activated charcoal (26 mg/ml) (NORIT, Amersfoort, The Netherlands) was added to adsorb free cAMP. The mixture was then centrifuged, and 200 μl of supernatant was counted in EcoLite (ICN Pharmaceuticals, Costa Mesa, CA) scintillation fluid.

Pertussis Toxin Pretreatment of Cells Assayed for cAMP. hDOR/CHO and hDOR/T1/CHO cells were pretreated for 18 to 24 h with either serum-free IMDM or 50 to 75 ng/ml pertussis toxin (List Biological, Campbell, CA) in serum-free IMDM. For experiments involving the superactivation pretreatment protocol, either IMDM or 100 nM SNC80 in IMDM was added to the appropriate wells for the final 4 h of pertussis toxin pretreatment. Cells were then washed once with fresh IMDM before the cAMP formation assay as described above.

IP_1 Formation. This was performed following a method adapted from Berridge and Irvine (1984). In 24-well plates, growth medium was aspirated and replaced with serum-free IMDM containing 0.2 μM myo-[^{3}H]inositol (PerkinElmer Life Sciences) for 18 h. The cells were washed in 1 ml fresh IMDM for 1 h. The medium was then replaced with IMDM containing 10 mM LiCl (Sigma Chemical, St. Louis, MO) for 10 min. Concentrations of SNC80 in IMDM were added for 1 h, then replaced with 0.5 ml of ice-cold methanol to

terminate the reaction. The wells were scraped and the contents were transferred to a chloroform water mixture (1 ml and 0.5 ml, respectively) for inositol phosphate extraction. After centrifugation, the extracts were loaded onto AG1-X8 resin (formate form) (Bio-Rad) and washed three times with water followed by three washes with a solution of sodium tetraborate and formic acid (5 mM and 60 mM, respectively) to elute free inositol. IP_1 was then eluted with a solution of formic acid and ammonium formate (0.1 M and 0.2 M, respectively). Eluent was counted in 16 ml of EcoLite scintillation fluid.

Pertussis Toxin Pretreatment of Cells Assayed for IP_1. In IP_1 formation experiments utilizing pertussis toxin, hDOR/CHO cells were pretreated with either 0.2 μM myo-[^{3}H]inositol in serum-free IMDM or 0.2 μM myo-[^{3}H]inositol with 50 ng/ml pertussis toxin in IMDM for the 18-h loading phase. Cells were then washed once and assayed for IP_1 formation as described above.

Data Analysis. Data were analyzed using Prism v3.02 (GraphPad, San Diego, CA) and are represented as mean ± S.E. To fit the data to a sigmoidal dose-response relationship, the following equation was used: $Y = \text{Bottom} + (\text{Top-Bottom}) / (1 + 10^{[(\text{LogEC}_{50} - X) \times \text{Hillslope})]}$, where X is the logarithm of the drug concentration and Y is the response. Statistical differences were ascertained by the Student's t test on the regression variables (top, bottom, logEC_{50}) as determined by a nonlinear regression analysis where the Hill coefficient was fixed to unity. In the inhibition of forskolin-stimulated cAMP formation experiments, results are normalized to 100% in the absence of SNC80 (top of the curve). In the adenylyl cyclase superactivation experiments, the overshoot in each cell line (comparison of the two curves representing each cell line pretreated with IMDM or SNC80) is normalized to 100% of maximal cAMP formation in the IMDM-pretreated cells (top of the curve for IMDM-pretreated cells). The basal adenylyl cyclase activities for the hDOR/CHO and hDOR/T1/CHO are close to 0 pmol of cAMP/million cells. The 10^{-4} M forskolin-stimulated value for hDOR/CHO is 307 ± 72 pmol of cAMP/million cells and 374 ± 180 pmol of cAMP/million cells for hDOR/T1/CHO cells. Using the Student's t test to compare these figures, a value of 0.67 is obtained, indicating no significant difference. Results of the IP_1 formation experiments are normalized to 100% of basal IP_1 formation. The basal IP_1 formation values for the two cell lines are 589 ± 460 cpm for the hDOR/CHO cells, and 1012 ± 90 cpm for the hDOR/T1/CHO cells. Using the Student's t test to compare these values, a value of 0.41 is obtained, indicating no statistically significant difference.

Results

The Effect of Pertussis Toxin on SNC80-Mediated IP_1 Formation in hDOR/CHO Cells. In hDOR/CHO cells, SNC80-mediated stimulation of IP_1 formation was sensitive to pertussis toxin (Fig. 1). SNC80 stimulated IP_1 formation with an EC_{50} value of 21 ± 13 nM and an E_{max} value of 311 ± 12%. After 18 h of pretreatment with 50 ng/ml pertussis toxin, hDOR/CHO cells showed no significant stimulation of IP_1 formation; consequently, no further statistics were analyzed on this data set.

The Effect of α-Transducin Expression in hDOR/CHO cells on SNC80-Mediated IP_1 Formation. Stable α-transducin expression in these cells resulted in a significant attenuation of maximal SNC80-mediated IP_1 formation. hDOR/CHO cells displayed an E_{max} value of 361 ± 17% of basal and hDOR/T1/CHO cells showed a maximum of 206 ± 9% of basal ($n = 3$; $p < 0.001$) This represents a significant attenuation of the E_{max} value by 59 ± 12% by the expression of α-transducin (Fig. 2).

The Effect of Pertussis Toxin Pretreatment on SNC80-Mediated Inhibition of Forskolin-Stimulated cAMP Formation in hDOR/CHO Cells. Pertussis toxin abated SNC80-mediated inhibition of forskolin-stimulated cAMP formation. SNC80 inhibited cAMP formation in untreated hDOR/CHO cells by 96 ± 9% with an EC_{50} value of 1.7 ± 1 nM, whereas there was no measurable inhibition after 50 to 75 ng/ml pertussis toxin pretreatment ($n = 3$; $p < 0.001$) (Fig. 3).

The Effect of α-Transducin Expression in hDOR/CHO Cells on SNC80-Mediated Inhibition of Forskolin-Stimulated cAMP Formation. Expression of α-transducin in hDOR/CHO cells (hDOR/T1/CHO cells) did not affect acute SNC80-mediated inhibition of forskolin-stimulated cAMP formation (Fig. 4). hDOR/CHO cells were inhibited 98 ± 11% and hDOR/T1/CHO cells were inhibited 97 ± 15%. The EC_{50} value for SNC80 was 1.6 ± 1 nM in hDOR/CHO cells and 1.6 ± 2 nM in hDOR/T1/CHO cells. There was no significant difference in these curves.

The Effect of Pertussis Toxin Pretreatment on Adenylyl Cyclase Superactivation in hDOR/CHO Cells. The cAMP overshoot is sensitive to pertussis toxin. In the absence of pertussis toxin, SNC80-pretreated cells displayed

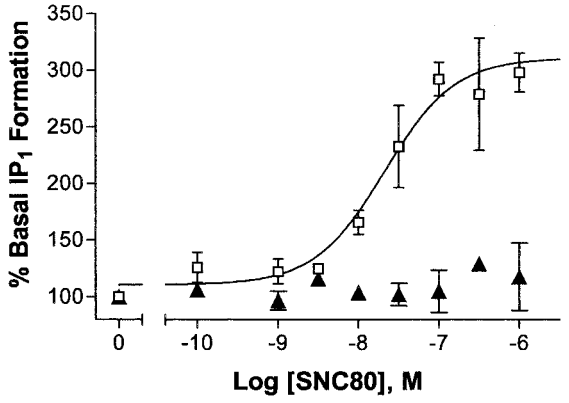

Fig. 1. Pertussis toxin sensitivity of SNC80-stimulated IP_1 formation. hDOR/CHO cells are stimulated by SNC80 to produce IP_1 with an EC_{50} value and maximum of 21 ± 13 nM and 311 ± 12% of basal, respectively (□, solid line, average of 11 independent experiments done in duplicate). In contrast, hDOR/CHO cells pretreated for 18 to 24 h with 50 ng/ml pertussis toxin (▲, average of 4 independent experiments done in duplicate) do not significantly stimulate IP_1 formation over basal as determined by nonlinear regression analysis.

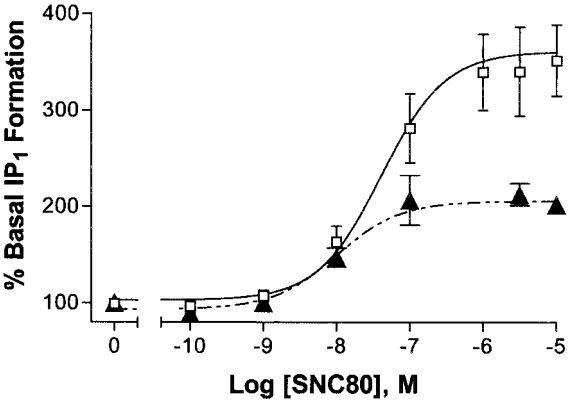

Fig. 2. SNC80-stimulated IP_1 formation in hDOR/CHO and hDOR/T1/CHO cells. Data are represented as percent of basal IP_1 formation for each cell line. Maximal IP_1 formation by hDOR/CHO (□, solid line, average of seven independent experiments done in duplicate) and hDOR/T1/CHO (▲, dashed line, average of three independent experiments done in duplicate) cells is 361 ± 17% and 206 ± 9%, respectively. The attenuation of the E_{max} by transducin is statistically significant ($p < 0.001$). The EC_{50} value of SNC80 in hDOR/CHO cells is 41 ± 40 nM and in hDOR/T1/CHO cells, 9.8 ± 10 nM.

a significant cAMP overshoot of 355 ± 165%, which was abolished by pertussis toxin pretreatment ($n = 10$; $p < 0.05$) (Fig. 5).

The Effect of α-Transducin Expression in hDOR/CHO Cells on Adenylyl Cyclase Superactivation. hDOR/CHO cells that were pretreated for 4 h with 100 nM SNC80, then washed three times, exhibited greater forskolin-stimulated cAMP formation than IMDM-treated control cells (Fig. 6). Forskolin-stimulated cAMP formation (10^{-4} M) in IMDM-pretreated cells was normalized to 100%. IMDM-pretreated cells exhibited an E_{max} value of 164 ± 8% ($n = 24$). SNC80-pretreated cells were significantly stimulated 376 ± 12% of IMDM-pretreated control, representing a cAMP overshoot of 229 ± 37% ($n = 22$; $p < 0.001$). The maximal forskolin-stimulated cAMP formation of CHO cells coexpressing the human δ-opioid receptor and α-transducin (hDOR/T1/CHO) was likewise normalized to 100% of 10^{-4} M forskolin-stimulated, IMDM-pretreated control cells. The E_{max} value of IMDM-pretreated hDOR/T1/CHO cells was 166 ± 14% and SNC80 pretreated hDOR/T1/CHO cells showed 163 ± 8% maximal stimulation ($n = 4$) (Fig. 6). The cAMP overshoot in the presence of α-transducin was not statistically significant.

Discussion

The human δ-opioid receptor stably expressed in CHO cells couples to inhibition of forskolin-stimulated adenylyl cyclase through $G_{\alpha i/o}$, and to activation of PLCβ, presumably through $G_{\beta\gamma}$. These $G_{\beta\gamma}$ subunits are derived from $G_{\alpha i/o}$ proteins, as evidenced by the complete block of the PLCβ response by pertussis toxin pretreatment. In addition, α-transducin expression in hDOR/CHO cells results in a decreased SNC80-stimulated IP_1 formation, which is evidence for scavenge of $G_{\beta\gamma}$ in this system. Concomitant with the attenuation of PLCβ_3 activation, a reduction in the cAMP overshoot is observed, without any change in SNC80-mediated acute inhibition of adenylyl cyclase. This indicates that α-transducin

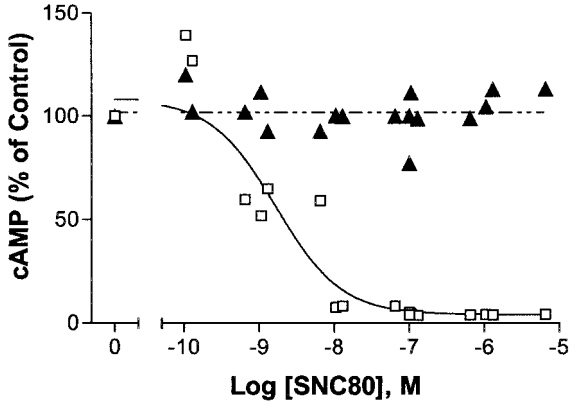

Fig. 3. Pertussis toxin sensitivity of SNC80-mediated inhibition of forskolin-stimulated cAMP formation. hDOR/CHO cells not pretreated with pertussis toxin (□, solid line) exhibit a dose-dependent inhibition of 0.1 mM forskolin-stimulated cAMP formation (EC_{50} = 1.7 ± 2 nM), whereas cells pretreated with 18 to 24 h of 50 to 75 ng/ml pertussis toxin (▲, broken line) show no measurable response (as determined by nonlinear regression analysis). Each line has been fitted to three independent experiments done in duplicate utilizing slightly different SNC80 concentrations.

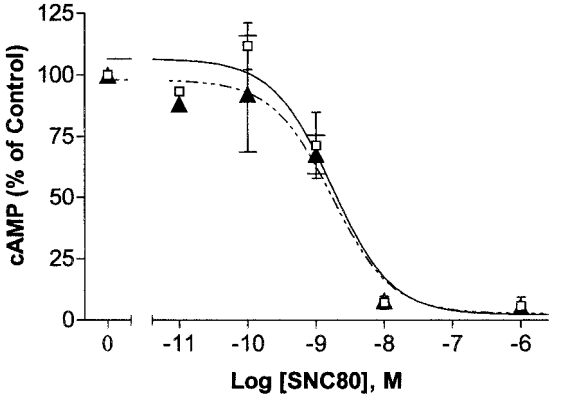

Fig. 4. SNC80-mediated inhibition of 0.1 mM forskolin-stimulated cAMP formation in hDOR/CHO (□, solid line) and hDOR/T1/CHO (▲, broken line) cells. hDOR/CHO cells were inhibited 98 ± 11% of control with an EC_{50} value of 1.6 ± 1 nM, whereas hDOR/T1/CHO cells were inhibited 97 ± 15% of control with an EC_{50} value of 1.6 ± 2 nM. There was no statistically significant difference in the E_{max} or EC_{50} values between the two cell lines. Each line is the average of three experiments done in duplicate.

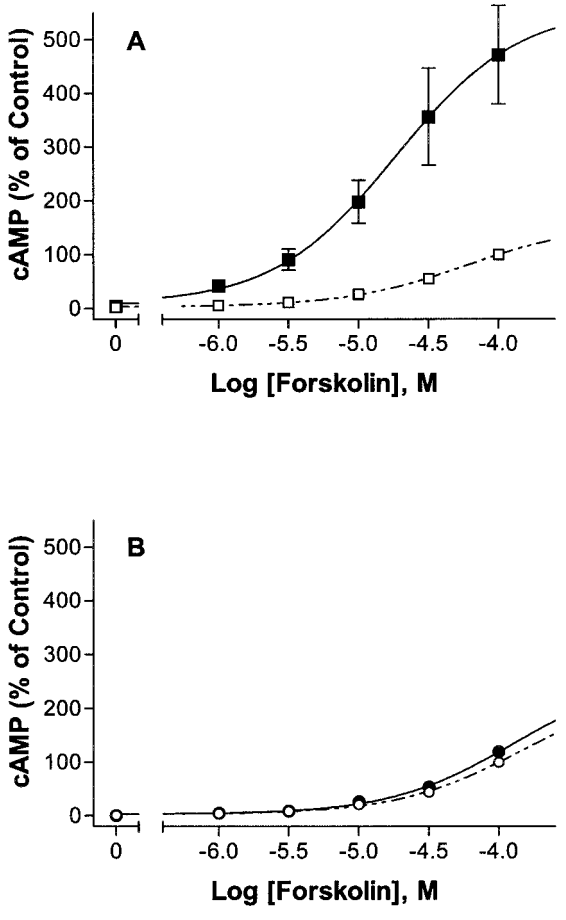

Fig. 5. Pertussis toxin sensitivity of the cAMP overshoot. A, the cAMP overshoot in hDOR/CHO cells not pretreated with pertussis toxin (□, broken line, IMDM-pretreated or ■, solid line, SNC80-pretreated) is statistically significant as ascertained by comparing the E_{max} values (157 ± 9% versus 558 ± 97%; $p < 0.001$). The EC_{50} values in the absence and presence of 4 h of 100 nM SNC80 are 59 ± 20 μM and 24 ± 20 μM, respectively. B, the cAMP overshoot after 18 to 24 h of 50 to 75 ng/ml pertussis toxin treatment (○, broken line, IMDM-pretreated or ●, solid line, SNC80-pretreated) is not significant (237 ± 39% versus 262 ± 38%; $p > 0.1$). The EC_{50} values in the absence and presence of 4 h of 100 nM SNC80 are 42 ± 100 μM and 74 ± 100 μM, respectively. Each line is the average of 10 individual experiments done in duplicate. Data are normalized to 100% of maximally-stimulated IMDM-treated cells.

selectively interferes with signaling through $G_{\beta\gamma}$ in hDOR/T1/CHO cells and that $G_{\beta\gamma}$ is required for AC superactivation.

The transfection of hDOR/CHO cells with α-transducin was successful (hDOR/T1/CHO), as evidenced by an increase in the intensity of a 41-kDa band over that found in untransfected hDOR/CHO cells and by functional studies demonstrating a novel coupling of α-transducin to the human δ-opioid receptor (using guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding; Varga et al., 2000). When the human δ-opioid receptor is stimulated by an agonist, $G_{i/o}$ proteins are activated, releasing activated $G_{\alpha i/o}$, and free $G_{\beta\gamma}$ subunits. Acute SNC80 fully inhibits forskolin-stimulated adenylyl cyclase via activated $G_{\alpha i/o}$ subunits, as this response is blocked by pretreatment with pertussis toxin (Fig. 3). Because no difference is observed in the acute inhibition of AC by SNC80 in the presence or absence of α-transducin (Fig. 4), α-transducin does not seem to interfere with δ-opioid receptor coupling to or signal transduction through $G_{\alpha i/o}$.

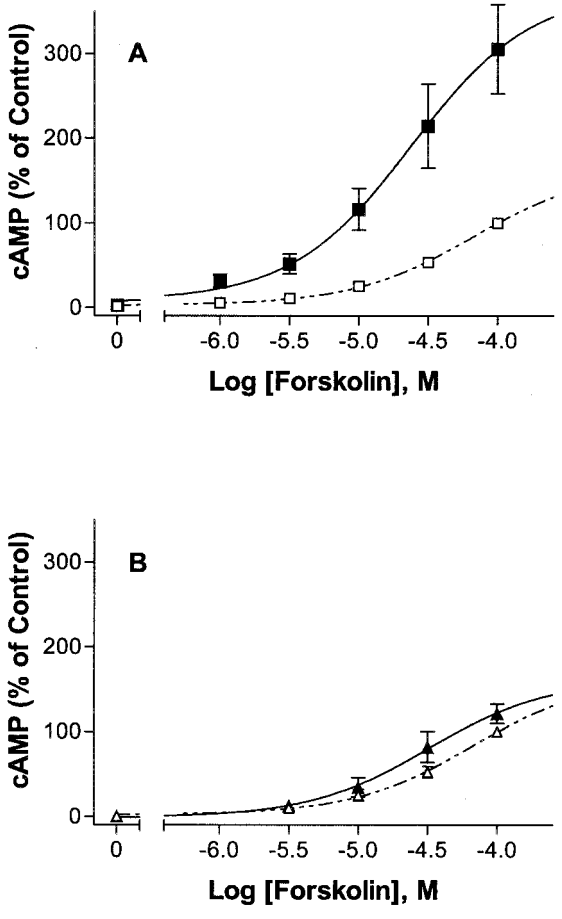

Fig. 6. Adenylyl cyclase superactivation in hDOR/CHO cells and hDOR/T1/CHO cells. A, the cAMP overshoot in the absence of α-transducin (□, broken line, IMDM-pretreated or ■, solid line, SNC80-pretreated) is statistically significant (164 ± 8% versus 376 ± 12%, $p < 0.001$). The EC_{50} values in hDOR/CHO cells in the absence and presence of 4 h 100 nM SNC80 are 14 ± 70 μM and 4.8 ± 20 μM, respectively. Each line represents the average of 22 independent experiments done in duplicate. B, the overshoot in the presence of transducin (▲, solid line, SNC80-pretreated or △, broken line, IMDM-pretreated) is not statistically significant (166 ± 14% versus 163 ± 8%; $p > 0.1$). Furthermore, the attenuation of maximal cAMP formation by transducin in the SNC80-pretreated groups (■ versus ▲) is significant ($p < 0.001$). The EC_{50} values in hDOR/T1/CHO cells in the absence and presence of 4 h 100 nM SNC80 are 29 ± 70 μM and 5.3 ± 30 μM, respectively. Each line represents the average of five independent experiments done in duplicate. Data are normalized to 100% of maximally-stimulated IMDM-treated cells.

α-Transducin presumably acts as a free $G_{\beta\gamma}$ scavenger and has been used as such on numerous occasions (Federman et al., 1992; Avidor-Reiss et al., 1996; Yoshimura et al., 1996; Selbie et al., 1997; Olianas and Onali, 1999). One method for measuring the $G_{\beta\gamma}$ scavenging ability of α-transducin is to determine the loss of coupling between $G_{\beta\gamma}$ and a known effector, such as the PLCβ_1 or PLCβ_3 enzyme, in the presence of α-transducin. Fortuitously, CHO cells have been shown to exclusively express the $G_{\beta\gamma}$-sensitive PLCβ_3: Dickenson and Hill (1998) demonstrated that of PLCβ isoforms 1 to 4, only the PLCβ_3 isoform is expressed in CHO cells (Dickenson and Hill, 1998). Evidence for the coupling of this enzyme to $G_{\beta\gamma}$ was provided by the observation that the pertussis toxin-sensitive [^{3}H]inositol phosphate accumulation by N^6-cyclopentyladenosine in CHO cells transfected with the adenosine A_1 receptor was attenuated in the presence of the $G_{\beta\gamma}$ scavenger, the C-terminal peptide of the β-adrenergic receptor kinase-1 (495–689). Furthermore, [D-Pen^2,D-Pen^5] enkephalin, a δ-opioid-selective agonist, has been shown to selectively activate PLCβ_3 (over PLC$\beta_{1,2,4}$) in a pertussis toxin-sensitive manner in intestinal smooth muscle via $G_{\beta\gamma}$ using anti-G_β and various anti-PLCβ antibodies to interrupt signaling (Murthy and Makhlouf, 1996). In our hDOR/T1/CHO cells, $G_{\beta\gamma}$ scavenge was demonstrated by the observed decrease in the maximal SNC80-mediated IP_1 formation in the presence of α-transducin (Fig. 2). The above observations substantiate the validity of the coupling of the human δ-opioid receptor to PLCβ_3 via $G_{\beta\gamma}$ in the heterologous CHO expression system used here and indicate that the hDOR/CHO system actually mirrors what is observed in a naturally occurring intact system.

Four-hour pretreatment with a maximally stimulating concentration of the full agonist, 100 nM SNC80, followed by removal of the agonist by repeated washes results in an increased AC activity. This response can be blocked by pretreatment with pertussis toxin (Fig. 5). These data indicate that $G_{\alpha i/o}$ subunits are required for AC superactivation. However, the $G_{\alpha i/o}$ subunits do not necessarily mediate the AC superactivation.

The significant attenuation of adenylyl cyclase (AC) superactivation by α-transducin expression clearly demonstrates the requirement for $G_{\beta\gamma}$ in this process (Fig. 6). Because both PLCβ_3 and AC superactivation are mediated by $G_{\beta\gamma}$, one possibility is that in the hDOR/CHO system, PLCβ_3 activation is upstream of the modification that results in the AC superactivation. This could occur through a number of pathways, but we have found that SNC80-induced ACVI [^{32}P] incorporation (Varga et al., 1999) follows a time course similar to that of AC superactivation. This suggests that AC phosphorylation in this system may be the cause of the superactivation. It is conceivable, therefore, that the IP_3 generated as a result of PLCβ_3 activation increases intracellular calcium through release from internal stores to activate calmodulin and calcium calmodulin-dependent kinase or that the diacyl glycerol generated activates protein kinase C. Further studies are required to identify a potential kinase and its role in AC superactivation.

Another possibility is that the requirement for $G_{\beta\gamma}$ in PLCβ_3 activation is independent of its requirement in AC superactivation. No consistent link between AC phosphory-

lation and AC superactivation has yet been found, which is in contradiction with the hypothesis in the preceding paragraph. Bayewitch et al. (1998) have demonstrated that ACVI, the only isoform of AC expressed in CHO cells (Varga et al., 1998) that has been shown to superactivate (Nevo et al., 1998), is directly inhibited by $G_{\beta\gamma}$ (Bayewitch et al., 1998), and that chronic treatment with agonist leads to modifications in either the structure of the $G_{\alpha i\beta\gamma}$ proteins or their compartmentalization (Bayewitch et al., 2000). Under these circumstances, the prediction is that there is constitutive $G_{\beta\gamma}$ inhibitory tone on AC, which is reversed upon chronic treatment with agonist, and leads to the aforementioned observed increase in the activity of AC and the cAMP overshoot (Vogel et al., 2000). Therefore, it would be useful to determine which site in the ACVI molecule interacts with $G_{\beta\gamma}$ subunits to inhibit its activity.

There is a subtle difference in the EC_{50} values for SNC80 between the stimulation of IP_1 formation (Figs. 1 and 2) and inhibition of forskolin-stimulated cAMP formation (Figs. 3 and 4). SNC80 seems to be about 10-fold less potent in stimulating PLCβ_3 than in inhibiting AC. One explanation for this is that the interaction of $G_{\alpha i/o}$ with AC is of higher potency than the interaction of $G_{\beta\gamma}$ with PLCβ_3 or that more $G_{\beta\gamma}$ is required to activate PLCβ than $G_{\alpha i/o}$ to inhibit AC.

In a previous study, we noted that the pertussis toxin-insensitive version of α-transducin used here was able to couple to the human δ-opioid receptor in hDOR/T1/CHO cells, as measured by an increased guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate binding (Varga et al., 2000). One consequence of such an interaction could be competition by α-transducin with endogenous G-proteins, thereby preventing any downstream effects mediated by endogenous G-proteins. This would serve to reduce PLCβ_3 activation and AC superactivation in much the same manner as a competitive antagonist at the receptor would. But at nanomolar potency, an acute inhibition of forskolin-stimulated AC activity is observed in the presence of α-transducin, which is identical to the inhibition seen in cells devoid of the scavenger (Fig. 4), which indicates that α-transducin is not interfering with signaling through endogenous $G_{\alpha i/o}$. Therefore, the results of the acute inhibition experiments suggest that α-transducin selectively interferes with $G_{\beta\gamma}$ signaling but has no effect on $G_{\alpha i/o}$ coupling.

We have demonstrated that coexpression of α-transducin with the human δ-opioid receptor diminishes the pertussis toxin-sensitive PLCβ activation by SNC80. This observation probably confirms the intended G-protein $\beta\gamma$ scavenging property of α-transducin. Furthermore, although α-transducin does not seem to have any affect on acute SNC80-mediated inhibition of AC, its expression prevents chronic SNC80-induced AC superactivation. This indicates that $G_{\beta\gamma}$ subunits are necessary for AC superactivation. By demonstrating the importance of $G_{\beta\gamma}$ in AC superactivation, we are providing greater insight into the molecular mechanism of what is understood to be a model for tolerance to and withdrawal from opioids and potentially other agonists acting at $G_{i/o}$-coupled receptors.

Acknowledgments

We thank Sue Waite for her help in the preparation of this manuscript and Michelle Thatcher and Carol Haussler for their extensive assistance with maintenance of the cell cultures.

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