Deltorphin II-induced Rapid Desensitization of δ-Opioid Receptor Requires Both Phosphorylation and Internalization of the Receptor

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Similar to other G protein-coupled receptors, rapid phosphorylation of the δ-opioid receptor in the presence of agonist has been reported. Hence, agonist-induced desensitization of the δ-opioid receptor has been suggested to be via the receptor phosphorylation, arrestin-mediated pathway. However, due to the highly efficient coupling between the δ-opioid receptor and the adenylyl cyclase, the direct correlation between the rates of receptor phosphorylation and receptor desensitization as measured by the adenylyl cyclase activity could not be established. In the current studies, using an ecdysone-inducible expression system to control the δ-opioid receptor levels in HEK293 cells, we could demonstrate that the rate of deltorphin II-induced receptor desensitization is dependent on the receptor level. Only at receptor concentrations ≤90 fmol/mg of protein were rapid desensitizations (t1/2 < 10 min) observed. Apparently, deltorphin II-induced receptor desensitization involves cellular events in addition to receptor phosphorylation. Mutation of Ser363 in the carboxyl tail of the δ-opioid receptor to Ala completely abolished the deltorphin II-induced receptor phosphorylation but not the desensitization response. Although the magnitude of desensitization was attenuated, the rate of deltorphin II-induced receptor desensitization remained the same in the S363A mutant as compared with wild type. Also, the S363A mutant could internalize in the presence of deltorphin II. Only when the agonist-induced clathrin-coated pit-mediated receptor internalization was blocked by 0.4 mM sucrose that the deltorphin II-induced receptor desensitization was abolished in the S363A mutant. Similarly, 0.4 mM sucrose could partially block the agonist-induced rapid desensitization in HEK293 cells expressing the wild type δ-opioid receptor. Taken together, these data supported the hypothesis that rapid desensitization of the δ-opioid receptor involves both the phosphorylation and the internalization of the receptor.

Within the proposed model by Lefkowitz and co-workers (1) for G protein-coupled receptor (GPCR),1 the step that initiates receptor desensitization involves the phosphorylation of the receptor by protein kinases including the G protein-coupled receptor kinases (GRKs), thereby promoting the recruitment of the cellular protein arrestin. Association of arrestin with the receptor enhances the uncoupling of the receptor from the respective G protein, thus blunting the signal transduction processes resulting in the receptor desensitization. The association of arrestin also appears to be critical in the agonist-induced, clathrin-coated vesicle-mediated receptor internalization (2). Arrestin also serves as an adaptor molecule in the β2-adrenergic receptor signaling such that a receptor-src kinase complex is formed through which activation of the mitogen-activated protein kinases Erk-1 and Erk-2 (Erk1/2) by the β2-adrenergic agonist is accomplished (3). Subsequent phosphorylation of GRKs and arrestin by the Erk1/2 serves as the feedback regulation of the activities of these proteins in the GPCRs signals transduction pathways (4, 5).

Being a member of the rhodopsin sub-family of the GPCRs, the mechanism of δ-opioid receptor desensitization could be similar to that of the β2-adrenergic receptor. There appears to be a casual relationship between the δ-opioid receptor phosphorylation and desensitization. Pei et al. (6) reported that the agonist DPDPDE-induced receptor phosphorylation could be potentiated by the co-expression of the GRK5 and was attenuated by the dominant negative GRK mutant. In the same study, the dominant negative GRK mutant blocked the DPDPDE-induced receptor desensitization. Likewise, overexpression of GRK2 and arrestin in HEK293 cells could accelerate the DPDPDE-induced δ-opioid receptor desensitization (7). Mutation of the last 4 Thr and Ser residues at the carboxyl tail of the receptor resulted in blockade of the GRK and arrestin-mediated desensitization in the Xenopus oocytes expressing the δ-opioid receptor (8). Overexpression of β-arrestin 1 alone resulted in the attenuation of the δ-opioid receptor activity (9). The desensitization of the endogenous δ-opioid receptor in the human neuroblastoma SK-N-BE cells was reported to correlate with the phosphorylation of the receptor (10). These studies supported the hypothesis of receptor phosphorylation as the mechanism for δ-opioid receptor desensitization.

However, the δ-opioid receptor lacking the C-terminal 31 amino acids, the sites for agonist-induced phosphorylation, can be rapidly desensitized by pretreating the CHO cells with DPDPDE for 10 min (11). The truncation of the δ-opioid receptor after Thr344 in the carboxyl tail domain of the receptor also resulted in the complete blockade of agonist-induced receptor phosphorylation in the HEK293 cells but not in the attenuation of the receptor internalization (12). These studies suggested that the putative arrestin-mediated events, the agonist-induced δ-opioid receptor desensitization and internalization, could occur in the absence of the agonist-induced receptor phosphorylation.

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1 The abbreviations used are: GPCRs, G protein-coupled receptors; GRKs, G protein-coupled receptor kinases; DPDPDE, [n-Pen2]-enkephalin; FACS, fluorescence-activated cell sorter; PA, ponasterone A; HA, hemagglutinin.

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In order to reconcile the reported observations, the role of receptor phosphorylation in the δ-opioid receptor desensitization must be clearly defined. There should be a direct correlation between the ability of the agonist to induce receptor phosphorylation and rapid desensitization. In a recent report, we have determined that the failure to correlate µ-opioid agonist-induced receptor desensitization, as measured by the regulation of adenylyl cyclase activity, was due to the rapid recycling and resensitization of the receptor (13). The rate of the receptor desensitization also appeared to be dependent on the receptor level expressed on the cell surface. Since the δ-opioid receptor is highly efficiently coupled to the adenylyl cyclase (14), the discrepancy in correlating the agonist-induced receptor phosphorylation and desensitization could stem from the presence of high receptor level expressed at the cell surface. Thus, the current studies were carried out to examine the role of receptor level in the δ-opioid agonist-induced receptor desensitization.

By using the edysosne-inducible expression system to control the expression of δ-opioid receptor in HEK293 cells, we could demonstrate a direct correlation between the receptor levels and the rates of agonist-induced receptor desensitization. Furthermore, we could demonstrate that the mechanism for agonist-induced rapid desensitization of the δ-opioid receptor involved both the phosphorylation and the agonist-induced internalization of the receptor.

**MATERIALS AND METHODS**

**Generating the S363A Mutant of δ-Opioid Receptor**—The site-directed mutagenesis of the δ-opioid receptor was carried out with the Altered Sites™ in vitro mutagenesis system provide by Promega Corp. (Madison, WI). DOR-1 was subcloned into the XhoI site of the phagemid pAlter-1, and single strand cDNA with complementary sequence of DOR-1 was then isolated accordingly. The mutagenesis of Ser363 to Ala was then accomplished using the oligodeoxynucleotide with the following sequence: 5'-CCGTTCCTTGCACCCCATCGTGGAAGC-3'. A PvuII endonuclease site was introduced at the Pro362 to Asp364 so as to facilitate the identification of the mutant from wild type receptor. After verifying the nucleotides sequence of the mutant by the dyeodeoxynucleotide sequencing using Sequenase II, the DOR-1 was excised by XhoI and subcloned in the pBKSα plasmid (Stratagene, La Jolla, CA). Then the EcoRI and XhoI fragment of the subsequent plasmid was excised and ligated to the DORTAG in pcDNA3 with the same fragment removed. DORTAG represents the subsequent plasmid was excised and ligated to the DORTAG in a gene, La Jolla, CA). Then the m

**Materials—**Expression vector, pLNDsp1, and reagents involved with the edysosne-inducible expression system, e.g. poronastone A, zecin, a-Pomastone A, were obtained from Delbeco’s modified Eagle’s medium and genicin (G-418) were purchased from Life Technologies, Inc. [3H]Diprenorphine (58 Ci/mmol) was supplied by Amer sham Pharmacia Biotech. [32P] (≥ 400 Ci/mmol) was supplied by ICS (Costa Mesa, CA). [32P]Acetylated cAMP (2200 Ci/mmol) was purchased from Linco Research Inc. (St. Charles, MO). Polyclonal antibodies for the cAMP RIA were developed by immunizing rabbits with succinyl cAMP conjugated to KLH. Mouse monoclonal anti-HA 1.1 clone 16B12 was purchased from Babco (Richmond, CA). Rat monoclonal anti-HA SF10 and mouse monoclonal anti-HA 12CA5 conjugated with peroxidase were purchased from Roche Molecular Biochemicals. Goat anti-mouse antibodies conjugated with Alexa 488 were purchased from Molecular Probes (Eugene, OR). Forskolin was purchased from Calbiochem. National Institute on Drug Abuse supplied the deltorphin II and other opioid ligands. All other chemicals were purchased from Sigma.

**RESULTS**

As with most GPCR, opioid receptor is rapidly phosphorylated upon the binding of the agonists (6, 7, 10, 17, 18). Though
During the short term agonist treatment. Only when the was sufficiently high enough to maintain the agonist activity the receptor on the cell surface that was not phosphorylated high level of receptor being expressed (13). The percentage of rylation and desensitization could be explained partially by the zation did not correlate with that of receptor phosphorylation of the d2 receptor, the rate of opioid receptor desensitization did not correlate with that of receptor phosphorylation (7). The discrepancy between the rates for receptor phosphorylation and desensitization could be explained partially by the high level of receptor being expressed (13). The percentage of the receptor on the cell surface that was not phosphorylated was sufficiently high enough to maintain the agonist activity during the short term agonist treatment. Only when the μ-opioid receptor level at the cell surface was relatively low (<50 fmol/mg protein) and the recycling of the receptor was blocked by monensin, then the rapid desensitization (in minutes) of the μ-opioid receptor was observed (13). Since the coupling of δ-opioid receptor to adenylyl cyclase is highly efficient (14), it is probable that the overexpression of the receptor is the reason for the failure to correlate the rates of δ-opioid receptor rapid desensitization and receptor phosphorylation (7).

In order to control the δ-opioid receptor level expressed on the cell surface and also eliminate any probable artifact due to position expression, the ecdysone-inducible system was used in current studies. The advantage of this system is that the insect steroid hormone analog, ponasterone A (PA), has been reported to induce the gene of interest 200-fold with no measurable effect on the mammalian cell physiology (19). Thus, the DORTAG and the Ser368 mutant of DORTAG (DORTAGS368A) were subcloned into the pINDsp1 vector containing the hybrid ecdysone response element (E/GRE) with multiple SP1 elements and transfected into HEK293 cells (EcR-293) expressing the heterotrimeric ecdysone receptor (VgEcR) and the retinoid X receptor. The EcR-293 cells surviving the G418 or DORTAGS368A in pINDsp1 vector were cultured in 100-mm plates in standard growth media containing various concentrations of ponasterone A. Afterward, the cells were harvested, and triplicate 1 nm [3H]diprenorphine binding was carried out in KRHB buffer in room temperature for 90 min. The nonspecific binding was determined in the presence of 10 μM naloxone. The values represent the averages from three separate ponasterone A-induced plates.

The opioid receptor level at the cell surface was relatively low, ~30 fmol/mg protein. In both cell lines, the receptor levels could be induced >100-fold at the highest concentration of PA used, 10 μM.

The opioid agonist and antagonist affinities for the receptor did not appear to be altered by the receptor level. As summarized in Table I, the antagonist diprenorphine affinities for the receptor were similar in both low and high level of δ-opioid receptor expressed. Furthermore, the affinities of the agonist deltorphin II for the receptor also were the same in the control EcR-293 cells and in cells treated with 1 μM PA. Most striking is the percentage of the receptor in the high affinity state was similar in EcR-293 cells expressing different levels of δ-opioid receptor (Table I). Since the ability to form high affinity complexes represents the interaction between the δ-opioid receptor and G proteins, these data indicate the receptor G protein coupling remains unchanged in the EcR-293 cells expressing the wide range of δ-opioid receptor.

As expected, the potency and maximal activity of deltorphin II were dependent on the level of δ-opioid receptor expressed in EcR-293 cells. As shown in Fig. 2, in the EcR-293 cells expressing the basal level of δ-opioid receptor, deltorphin II was able to elicit 32 ± 1.2% maximal inhibition of the adenylyl cyclase activity. The potency of deltorphin II to produce 50% of maximal response, 1.8 ± 0.2 nM, was similar to the high affinity binding of the peptide to the receptor, 1.3 ± 0.5 nM. Thus, in the EcR-293 cells with basal level of receptor expression, the maximal opioid effect required the full occupancy of the receptor. However, when the EcR-293 cells were treated with 5 μM PA for 48 h, an increase in both the potency and maximal activity of deltorphin II was observed (Fig. 2). The increase in potency and maximal response of deltorphin II was PA concentration-dependent. As summarized in Table II, there was a pronounced increase in the potency of deltorphin II when the δ-opioid receptor level was increased by 3-fold in the presence of 0.1 μM PA. There was no further increase in the potency of deltorphin II in EcR-293 cells treated with PA concentration >0.5 μM for 48 h. There was also no parallel increase in the maximal activity of deltorphin II in EcR-293 cells treated with 0.5 μM or higher concentrations of PA, although there was a 2-fold difference in the receptor level being induced. Hence, as predicted by the classical receptor theory involving amplification of the signals, there is a critical δ-opioid receptor concentration involved in receptor signalling above which increase in the active receptor concentration will not alter both the efficacy and potency of the agonist.

When these EcR-293 cells were treated with deltorphin II, a rapid phosphorylation of the receptor was observed. As summarized in Fig. 3A, 1 μM deltorphin II produced an apparent

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**Table I**

Relative affinities of opioid ligands at different levels of δ-opioid receptor expressed in EcR-293 cells

| Ponasterone A | [3H]Diprenorphine binding | Deltorphin II, Kᵢ |
|--------------|---------------------------|-------------------|
| μM           | nM                        | pmol/mg-protein   | nM          |
| 0            | 0.29 ± 0.12               | 0.03 ± 0.02       | 1.3 ± 0.3   |
|              |                           |                   | (79 ± 5%)    |
|              |                           |                   | 540 ± 380   |
| 1            | 0.61 ± 0.05               | 3.9 ± 0.09        | 2.5 ± 0.5   |
|              |                           |                   | (70 ± 1%)    |
|              |                           |                   | 200 ± 60    |

EcR-293 cells transfected with the DORTAGpINDsp1 were treated with ponasterone A for 48 h. Afterward, the membranes were prepared, and the saturation and competition binding experiments were carried out as described (7). The relative affinities and maximal binding level were then calculated using the GraphPad program. The values in parentheses represent the percentage of receptor in the high affinity state.

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**Fig. 1** Ponasterone A concentration-dependent induction of the δ-opioid receptor. EcR-293 cells stably expressing the DORTAG or DORTAGS368A in pINDsp1 vector were cultured in 100-mm plates in standard growth media containing various concentrations of ponasterone A. Afterward, the cells were harvested, and triplicate 1 nm [3H]diprenorphine binding was carried out in KRHB buffer in room temperature for 90 min. The nonspecific binding was determined in the presence of 10 μM naloxone. The values represent the averages from three separate ponasterone A-induced plates.
Mechanism of δ-Opioid Receptor Desensitization

**Fig. 2. Dependence of deltorphin II inhibition of adenylyl cyclase activity on ponasterone A.** EcR-293 cells expressing the DORTAG were treated with 0 (○) or 5 μM (●) PA for 48 h. Afterward, the abilities of various concentrations of deltorphin II to inhibition of 10 μM forskolin-stimulated adenylyl cyclase activity in these cells were determined. The dose-responses curves were then analyzed and fitted with the GraphPad program. The values represent the average ± S.D. of three separate dose-response curves.

Transient increase in the phosphorylation of the δ-opioid receptor, with maximal phosphorylation at 10 min after addition of agonist. The δ-opioid receptor maximally phosphorylated at 10 min was independent of the level of receptor being induced by PA (data not shown). With prolonged incubation with deltorphin II, there was a decrease in the apparent receptor phosphorylation level. However, this apparent decrease in receptor phosphorylation was due to the rapid decrease in the total cellular receptor protein content, as indicated by Western analysis (Fig. 3B). When multiple experiments were summarized and the levels of receptor phosphorylation were normalized with the receptor Western analysis, a sustained deltorphin II-induced receptor phosphorylation was observed during 60 min of incubation (Fig. 3C). However, the deltorphin II-induced δ-opioid receptor desensitization proceeded in a much slower rate (Fig. 4). The \( t_{1/2} = 135 ± 73 \) min for the desensitization rate was observed, which was significantly slower than the rate of receptor phosphorylation. In the case of the μ-opioid receptor, the rate of agonist-induced receptor desensitization was increased if the recycling of the receptor was blocked by monensin (13). As shown in Fig. 4, 50 μM monensin also could increase the rate of δ-opioid receptor desensitization. Although the \( t_{1/2} \) for the agonist-induced receptor desensitization was increased to 62.3 ± 13 min in the presence of monensin, the rate still was much slower than that observed with receptor phosphorylation.

As indicated with the PA concentration-dependent studies, the potency and maximal activity of deltorphin II were not affected by the active receptor density until the receptor concentration was decreased past a critical level (Table II). Hence, the failure to observe rapid desensitization of the δ-opioid receptor was due to the relatively high receptor level being induced. When the EcR-293 cells were treated with various concentrations of PA, a direct correlation between the δ-opioid receptor levels and the rates of receptor desensitization was observed (Fig. 5). The \( t_{1/2} \) values, determined from the experiments summarized in Fig. 5, correlated with the relative receptor levels induced in these EcR-293 cells (Table III). At the basal level of the δ-opioid receptor expressed in EcR-293 cells, in the presence of monensin, 1 μM deltorphin II induced a rapid desensitization of the receptor with a \( t_{1/2} \) value of 9 min. In contrast, the desensitization rate was significantly lower with the δ-opioid receptor concentration >1.5 pmol/mg protein, \( t_{1/2} >60 \) min (Table III). Although in EcR-293 cells treated with 0.5 μM PA the \( t_{1/2} \) value increased by 2-fold only with a 30-fold increase in the receptor level, a complete loss in the ability of δ-opioid receptor to inhibit the adenylyl cyclase was not observed (Fig. 5). Hence, by limiting the receptor concentration at the cell surface, a rapid rate for δ-opioid receptor desensitization similar to that of the receptor phosphorylation can be demonstrated.

With the ability to observe rapid desensitization, we can now address the issue of whether receptor phosphorylation is the mechanism for the blunting of δ-opioid receptor signaling. In a separate study, we determined that by mutating the Ser
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 in the carboxyl tail domain of the δ-opioid receptor to Ala, the [d-Pen
4,5]
 enkephalin (DPDPE)-induced receptor phosphorylation was completely eliminated. Thus, we subcloned the DORTAGS363A into the pINDsp1 vector and established an EcR-293 cell line stably expressing this δ-opioid receptor mutant. As shown in Fig. 1, the expression of this mutant receptor binding was PA concentration-dependent also. However, the maximal level of receptor induced was 50% of the wild type at the high PA concentration tested. Similar to the EcR-293 cells expressing the wild type receptor, the potency and maximal activity of deltorphin II in EcR-293 cells expressing the DORTAGS363A was dependent on the PA concentration used in inducing the receptor (Table II). Thus, the mutation of this serine moiety did not affect the pharmacological profile of the receptor at various PA concentrations.

Similar to our observation with DPDPE, deltorphin II did not induce phosphorylation of the δ-opioid receptor S363A mutant (Fig. 6, A and B). If the phosphorylation is a prerequisite for the observed deltorphin II-induced rapid desensitization of the receptor, then the elimination of phosphorylation should eliminate the receptor desensitization. This was not the case. The EcR-293 cells expressing either the wild type or the mutant receptor were treated with 0.1 or 0.2 μM of PA, respectively. These concentrations of PA were chosen because similar levels of wild type and mutant receptors were expressed subsequently (Fig. 1). As summarized in Fig. 6C, deltorphin II could induce the rapid desensitization of the δ-opioid receptor S363A mutant. Although the system was only 35% desensitized in the EcR-293 cells expressing the mutant receptor as compared with 100% desensitized in the cells expressing the wild type receptor, the rates of desensitization were similar. The \( t_{1/2} \) value for the DORTAGS363A mutant was determined to be 15.3 ± 4.7 min as compared with the \( t_{1/2} \) value of 9.9 ± 1.6 min for the wild type receptor. Hence, the elimination of the agonist-induced receptor phosphorylation reduced the magnitude of desensitization but not the rate of desensitization.

It is possible that the observed difference in the level of desensitization between the EcR293 cells expressing the wild type and S363A mutant receptor is in the rate of degradation of the receptor. As shown in Fig. 3B, deltorphin II treatment resulted in a rapid decrease in the total receptor content. This observation is in agreement with that reported by Tsao and von Zastrow (20). However, this appeared to be not the mechanism. During the prolonged deltorphin II treatment, monensin was included in the medium. The decrease in the total cellular receptor content during agonist treatment was blunted (Fig. 7). In the absence of monensin, 1 h of 1 μM deltorphin II treatment resulted in 60.5 ± 2.8 and 61 ± 7.4% reduction in the total cellular content of wild type and mutant receptor, respectively. In the presence of 50 μM monensin, the reduction in cellular receptor content was decreased to 25.5 ± 2.5 and 33.6 ± 5.8% reduction in the wild type and mutant receptor (Fig. 7). The

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DORTAG and DORTAGS363A expressed in EcR293 were induced with various concentrations of ponasterone A for 48 h as described under "Materials and Methods." The abilities of various concentrations of deltorphin II to inhibit the forskolin-stimulated adenylyl cyclase activities were determined. The IC₅₀ and maximal inhibition values were then calculated by the analysis of the dose-response curves with GraphPad prism program. The values represent the average ± S.D. of a minimum of three different experiments, and the values in parentheses represent the amount of 2 nM [³H]diprenorphine specifically bound to cells treated with ponasterone A.

| Ponasterone A | DORTAG | Maximum inhibition | IC₅₀ | Maximum inhibition | DORTAGS363A |
|--------------|--------|------------------|-----|-------------------|-------------|
| µM | nM | % | nM | % |
| 0 | 1.8 ± 0.2 | 32 ± 1.2 (0.027 ± 0.005) | 1.5 ± 1.7 | 30.3 ± 4.6 (0.029 ± 0.009) |
| 0.1 | 0.77 ± 0.11 | 53.8 ± 0.60 (0.087 ± 0.013) | 1.5 ± 3.0 | 39.8 ± 8.4 (0.074 ± 0.006) |
| 0.2 | 0.11 ± 0.03 | 71.0 ± 2.9 (0.092 ± 0.095) | (2.24 ± 0.096) |
| 1 | 0.11 ± 0.02 | 78.4 ± 1.2 (1.45 ± 0.033) | (1.58 ± 0.13) |
| 2 | 0.12 ± 0.03 | 80.7 ± 1.8 (1.60 ± 0.033) | (1.58 ± 0.13) |
| 5 | 0.07 ± 0.04 | 74.7 ± 3.1 (2.24 ± 0.096) | (1.58 ± 0.13) |

continued decrease in total cellular receptor content in the presence of monensin could be due to the removal of PA during the assay, reflecting the normal turnover of the δ-opioid receptor. These data suggested the observed difference in receptor desensitization could not be accounted for by the difference in total receptor content among the EcR-293 cells expressing the wild type and mutant receptor.

One mechanism which the DORTAGS363A could be desensitized by deltorphin II pretreatment is that the receptor is internalized in the presence of the agonist. When FACS analysis was used to determine the agonist-induced receptor internalization in the EcR-293 cells expressing the wild type and mutant receptors, deltorphin II induced a time-dependent loss of cell surface fluorescence in cells expressing the wild type as well as those expressing the mutant receptors (Fig. 8). Again, the calculated t¹₂ values for the maximal receptor internalization of these two receptors were very similar. The t¹₂ for the wild type was determined to be 12.1 ± 0.06 min, while the t¹₂ for the S363A mutant was determined to be 15.5 ± 0.6 min. However, there was a significant reduction in the receptor level being internalized. In EcR-293 cells expressing the wild type receptor, 1 µM deltorphin II could induce the internalization of 89 ± 0.1% of the receptor. On the other hand, the same concentration of the deltorphin II could only induce 39 ± 0.6% of the DORTAGS363A receptor (Fig. 8). This difference in the agonist-induced receptor internalization could not be due to the differences within the receptor level expressed in these two cell lines. For the rate and the magnitude of wild type receptor being internalized were independent of the PA concentration used to induce the expression of receptor in the EcR-293 cells (data not shown). Thus, the absence of receptor phosphorylation did not decrease the rate but did decrease the magnitude of receptor being internalized in the presence of agonist.

The ability of deltorphin II to induce a rapid albeit lower magnitude of receptor internalization in EcR-293 cells expressing the δ-opioid receptor S363A mutant suggests this pathway might participate in the agonist-induced rapid desensitization of the receptor. Internalization of the δ-opioid receptor has been demonstrated to involve the clathrin-coated pits-mediated, arrestin- and dynamin-dependent pathway (12, 21). Blockade of this pathway with 0.4 M sucrose would prevent the internalization of the receptor. As shown in the Fig. 9A, 0.4 M sucrose completely blocked the deltorphin II-induced δ-opioid receptor internalization in the EcR-293 cells. When the agonist treatment of the cells was carried out in the presence of 0.4 M sucrose, reduction in the magnitude of agonist-induced rapid desensitization of the δ-opioid receptor was observed (Fig. 9B).

In EcR-293 cells expressing the wild type δ-opioid receptor, instead of complete loss of activity, 30 min of 1 µM deltorphin II pretreatment in the presence of 0.4 M sucrose reduced 77 ± 2.2% of the initial activity of the receptor. These data suggested that 23% of the observed rapid desensitization of the δ-opioid receptor was due to internalization of the receptor. The involvement of receptor internalization in the rapid desensitization process could be demonstrated with EcR-293 cells expressing the DORTAGS363A mutant. Treatment of the cells with 0.4 M sucrose completely blocked the ability of the 1 µM deltorphin II to induce rapid desensitization of the δ-opioid receptor (Fig. 9B). The ability of 1 µM deltorphin II to inhibit the adenylyl cyclase activity after 30 min of agonist pretreatment in the presence of sucrose was determined to be 103 ± 4.6% of the control. Hence, the residual desensitization in the absence of receptor phosphorylation observed in EcR-293 cells expressing the δ-opioid receptor S363A mutant must be due to the agonist-induced internalization of the receptor.

**DISCUSSION**

Agonist-induced phosphorylation of receptors has been demonstrated unequivocally as the key step in the blunting of many GPCRs’ signaling. Examples of GPCRs that are desensitized by such mechanisms are the β₂-adrenergic (1), muscarinic (22), PGE EP2 and EP4 (23, 38), adenosine A1 and A3 (24, 25), somatostatin (26, 27), neurokinin-2 (28), and secretin (29) receptors, among many. Opioid receptor has been suggested to belong to this group of GPCRs. Data in support of the hypothesis that receptor phosphorylation is the key step for agonist-induced opioid receptor desensitization are mainly from the overexpression of GRKs or arrestin (6, 8, 9). Mutation of the putative phosphorylation sites resulting in the alteration of the...
agonist-induced receptor desensitization also supported this hypothesis (30). However, a direct correlation between the receptor phosphorylation and agonist-induced rapid desensitization of the receptor has been controversial. Not only the rates of desensitization of both μ- and δ-opioid receptors were significantly slower than that of receptor phosphorylation rates (7), but also receptor desensitization could not be blocked by the removal of putative phosphorylation sites within the carboxyl tail domains of these receptors (11, 31). Furthermore, arrestin was not recruited by the morphine-induced, phosphorylated δ-opioid receptor in HEK293 cells overexpressing GRK2 (32). These observations raised the question of whether receptor phosphorylation is the key for opioid receptor rapid desensitization.

In an earlier study, we have investigated the role of μ-opioid receptor phosphorylation in its desensitization (13). The ability of μ-opioid receptor to be recycled and resensitized has been reported by Koch et al. (32). Thus, with relatively high receptor phosphorylation rates...

**FIG. 3.** Time course of deltorphin II-induced phosphorylation of δ-opioid receptor. EcR-293 cells expressing the DORTAG were treated with 2.5 μM ponasterone A for 48 h. The cells were then labeled with 32P, treated with 1 μM deltorphin II for various time intervals, and the degrees of receptor phosphorylation were determined and quantified as described under “Materials and Methods.” A, represents the phosphoreceptor bands corresponding to the δ-opioid receptor at different time of deltorphin II treatment, as indicated. B, the gel represented in A was electroblotted on PVDF membrane, and Western analysis was carried out with the peroxidase-conjugated monoclonal antibodies (12CA5) to the hemagglutinin epitope tag. The bands from the phosphorimage in A and the receptor protein bands from the Western analysis in B were quantitatively analyzed. The degrees of δ-opioid receptor phosphorylation at different times of deltorphin II treatment were then normalized to the receptor protein content and are summarized in C. The values represent the averages from two phosphorylation experiments.

**FIG. 4.** Rate of δ-opioid receptor desensitization in the presence of deltorphin II. EcR-293 cells expressing the δ-opioid receptor were treated with 1 μM ponasterone A for 48 h and were exposed to 1 μM deltorphin II for various amounts of time. Then the ability of 1 μM deltorphin II to inhibit the 10 μM forskolin-stimulated adenyl cyclase activity after various times of agonist treatment in the absence (○) or presence (●) of 50 μM monensin was determined. The values represent the average ± S.D. of three separate experiments.

**FIG. 5.** Ponasterone A concentration-dependent decrease in the rates of deltorphin II-induced δ-opioid receptor desensitization. EcR-293 cells expressing the δ-opioid receptor were treated with various concentrations of ponasterone A for 48 h. The rates of deltorphin II-induced receptor desensitization were then determined as described under “Materials and Methods.” The curves were then fitted and t1/2 values calculated with the GraphPad program. The values represent average ± S.D. from 3 to 5 separate rate experiments.
density, in conjunction to the high efficiency of coupling between receptor and adenylyl cyclase (14), loss of receptor activity would not be observed unless the receptor level has decreased past the critical level. We demonstrated that the blockade of \( m \)-opioid receptor recycling, and hence receptor resensitization, would result in a rapid desensitization of the receptor when the receptor density was limited (13). Our current approach was to determine the rate of receptor desensitization in the absence of receptor phosphorylation.

The rates in which 1 \( \mu \)M deltorphin II lost its ability to inhibit the forskolin-stimulated adenylyl cyclase activity in EcR293 cells pretreated with various concentrations of ponasterone A for 48 h were calculated from the data summarized in Fig. 5. The amount of \( m \)-opioid receptor expressed in the EcR293 cells at these ponasterone A concentrations was determined with 2 nM \(^{3}H\) diprenorphine binding studies.

**TABLE III**

Rate of deltorphin II-induced \( m \)-opioid receptor desensitization is dependent on receptor density

| Ponasterone A \( \mu \)M | Receptor density \( \text{pmol/mg protein} \) | Rate of desensitization, \( t_{1/2} \) (min) |
|--------------------------|---------------------------|------------------|
| 0                        | 0.034 ± 0.032             | 9.6 ± 3.0        |
| 0.05                     | 0.048 ± 0.028             | 8.9 ± 1.0        |
| 0.1                      | 0.087 ± 0.013             | 9.9 ± 1.6        |
| 0.5                      | 0.92 ± 0.096              | 19.9 ± 3.6       |
| 1                        | 1.48 ± 0.033              | 62.2 ± 15.2      |
| 2                        | 1.6 ± 0.033               | 68.2 ± 16.2      |
| 5                        | 2.24 ± 0.096              | 98.5 ± 45.6      |

**FIG. 6.** Ability of deltorphin II to induce receptor desensitization in the absence of receptor phosphorylation. DORTAGS363A in pINDsp1 was stably expressed in EcR-293 cells. In the upper panel, the cells were treated with 5 \( \mu \)M PA for 48 h and then the ability of 10 min deltorphin II (1 \( \mu \)M) treatment to induce receptor phosphorylation was then determined. The ability of 10-min deltorphin II treatment to induce phosphorylation of the wild type \( m \)-opioid receptor was also carried out in the same experiments. A, lanes 1 and 2 represent the degree of wild type \( m \)-opioid receptor being phosphorylated in the absence and presence of 1 \( \mu \)M deltorphin II, respectively. Lanes 3 and 4 present the phosphorylation of S363A mutant in absence and presence of deltorphin II, respectively. B represents the Western analysis of the same gel shown in A with the horseradish peroxidase-conjugated antibody to the hemagglutinin epitope tag as described in the legend of Fig. 3. A represents the actual immunoblots of the \( m \)-opioid receptor from one of the experiments. B represents the average ± S.D. of the Western analysis from two separate experiments.

**FIG. 7.** Western analysis of the total cellular content of \( m \)-opioid receptor after deltorphin II treatment. EcR-293 cells expressing the wild type [■] or S363A mutant [■] \( m \)-opioid receptor were treated with 2 \( \mu \)M PA for 48 h. The cells were then exposed to 1 \( \mu \)M deltorphin II for 1 h in the presence or absence of 50 \( \mu \)M monensin. Afterward, the cells were harvested and solubilized, and Western analysis of the total receptor content was carried out with the peroxidase-conjugated monoclonal antibodies (12CA5) to the hemagglutinin epitope tag as described in the legend of Fig. 3. A, lanes 1 and 2 represent the degree of wild type \( m \)-opioid receptor being phosphorylated in the absence and presence of deltorphin II, respectively. B represents the Western analysis of the same gel shown in A with the horseradish peroxidase-conjugated anti-HA monoclonal antibody (12CA5) to the hemagglutinin epitope tag as described in the legend of Fig. 3. A represents the actual immunoblots of the \( m \)-opioid receptor from one of the experiments. B represents the average ± S.D. of the Western analysis from two separate experiments.

**FIG. 8.** Deltorphin II-induced receptor internalization in EcR-293 cells expressing either DORTAG or DORTAGS363A. EcR-293 cells expressing the wild type [■] or S363A mutant [■] \( m \)-opioid receptors were treated with 5 \( \mu \)M PA for 48 h. One hour prior to exposing the cells to 1 \( \mu \)M deltorphin II for various times, 50 \( \mu \)M monensin was added to the cells. Then the amount of receptor being internalized was determined with FACS analysis. The values represent the average ± S.D. of 3 experiments.

creased past the critical level. We demonstrated that the blockade of \( \mu \)-opioid receptor recycling, and hence receptor resensitization, would result in a rapid desensitization of the receptor when the receptor density was limited (13). Our current approach was to determine the rate of receptor desensitization in the absence of receptor phosphorylation.
Mechanism of δ-Opioid Receptor Desensitization

3–6 experiments. This was clearly demonstrated with the receptor at the cell surface did not result in parallel decrease in the fraction of active receptor (Table III). The reduction in the fraction of active receptor also was dependent on the receptor density. With the presence of 0.4 M sucrose, afterward, the amount of the wild type δ-opioid receptor being internalized in the presence of agonist in the absence or in the presence of 0.4 M sucrose was then determined by FACS analysis. The ability of 1 μM deltorphin II to inhibit the forskolin-stimulated adenyl cyclase in the absence or in the presence (34) of agonist pretreatment was also measured. The values represent the average ± S.D. from 3–6 experiments.

Fig. 9. Effect of 0.4 M sucrose on the deltorphin II-induced δ-opioid receptor desensitization in EcR-293 cells expressing either DORTAG or DORTAGS363A. EcR-293 cells stably transfected with either DORTAG or DORTAGS363A in pINDsp1 were treated with 0.1 μM or 0.2 μM PA respectively for 48 h. The cells were then pretreated with 1 μM deltorphin II for 30 min in the absence or in the presence of 0.4 M sucrose. Afterward, the amount of the wild type δ-opioid receptor being internalized in the presence of agonist in the absence (C) or in the presence (D) of sucrose was then determined by FACS analysis (A). The ability of 1 μM deltorphin II to inhibit the forskolin-stimulated adenyl cyclase in the absence (C) or in the presence (D) of agonist pretreatment was also measured (B). The values represent the average ± S.D. from 3–6 experiments.

Download Fig. 9

The ability to detect agonist-induced rapid desensitization of the receptor allows us to examine the question whether receptor phosphorylation is the key for this cellular adaptational event. In a separate study, we were able to block completely the DPDPE-induced receptor phosphorylation by a single amino acid mutation of the Ser363 to Ala. Whether this serine residue is a phosphorylation site itself remains to be resolved. As shown in the current study, this S363A mutation also blocked the deltorphin II-induced phosphorylation of the δ-opioid receptor (Fig. 6). Nevertheless, even without agonist-induced receptor phosphorylation, pretreatment of the EcR-293 cells with deltorphin II resulted in a similar rate but reduction in the magnitude of receptor desensitization (Fig. 6). These differences in the magnitudes of desensitization among the wild type and mutant receptor could not be due to the differences in the receptor degradation rates, as indicated by the similarity in the receptor levels (Fig. 7). The ability to desensitize in the absence of phosphorylation suggests the observed blockade of the δ-opioid receptor desensitization with GRK dominant negative mutant could not be simply due to the abolition of receptor phosphorylation (6). The ability to desensitize at the same rate in the absence of receptor phosphorylation also suggests the overexpression of GRK should increase the magnitude but not the rate of δ-opioid receptor desensitization. Furthermore, the blockade of receptor internalization with 0.4 M sucrose resulted in residual agonist activity instead of complete desensitization suggested alternate mechanism for δ-opioid receptor desensitization in addition to the binding of arrestin to phosphorylated receptor and subsequent uncoupling of the receptor from the G protein. Receptor uncoupling appears to account for 65–77% of the rapid desensitization, as indicated by wild type desensitization in the presence of sucrose thus blocking internalization, and by the S363A mutant where receptor phosphorylation was eliminated. Receptor internalization plays a role in the agonist-induced rapid desensitization of the δ-opioid receptor, as suggested by Pak et al. (34) to be the mechanism for the μ-opioid receptor desensitization in the CHO cells.

The ability for the agonist to induce internalization of the non-phosphorylated δ-opioid receptor via the dynamin-dependent pathway has been reported (12). Overexpression of arrestin enhanced the morphine-induced internalization and desensitization of the μ-opioid receptor (35). Since morphine does not appear to induce phosphorylation of the μ-opioid receptor (17, 36), the ability of the arrestin to interact with non-phosphorylated opioid receptor could trigger the internalization of the receptor. Our current studies also indicated that the non-phosphorylated S363A mutant of the δ-opioid receptor internalized in the presence of deltorphin II (Fig. 8). Whether the agonist-induced association of arrestin with this non-phosphorylated receptor remains to be demonstrated. Nevertheless, a reduction in the magnitude of receptor being internalized suggested agonist-induced receptor phosphorylation contributed to this arrestin-mediated event. Phosphorylation of the receptor is not the obligatory event for the agonist to induced δ-opioid receptor internalization. The probable association of arrestin with the non-phosphorylated δ-opioid receptor triggered the receptor internalization and not the uncoupling of the receptor from G protein.
proteins. For the deltorphin II induced rapid desensitization of the S363A mutant of \(\delta\)-opioid receptor could be blocked completely only if the clathrin-coated vesicle-mediated receptor internalization was abolished by 0.4 M sucrose (Fig. 9). Thus, it is also probable that arrestin interacted at multiple sites of the \(\delta\)-opioid receptor. Elimination of the receptor phosphorylation prevented the arrestin to interact with sites that were critical for G protein-receptor uncoupling. If the observed internalization of S363A was via the arrestin/dynamin pathway, then the elimination of receptor phosphorylation by this mutation did not abolish the arrestin interaction with sites that were critical for the agonist-induced receptor internalization.

The rapid desensitization of \(\delta\)-opioid receptor in the presence of agonist appears to involve multiple pathways. Our current studies indicated that \(\delta\)-opioid receptor rapid desensitization involved both the agonist-induced phosphorylation of the receptor and the subsequent rapid internalization of the receptor. Unlike other GPCR such as the somatostatin receptor (37), receptor desensitization cannot be due to internalization of the \(\delta\)-opioid receptor alone. This was clearly demonstrated by the experiments in which the blockade of the clathrin-coated pits-mediated internalization of the wild type receptor only resulted in the partial attenuation of the rapid desensitization (Fig. 9). The dynamic cycle of the receptor phosphorylation, internalization, and desensitization definitely contributes to the rate of the agonist-induced receptor desensitization. In situations when overexpression of GRK and \(\beta\)-arrestin could increase the rates of agonist-induced \(\delta\)-opioid receptor desensitization (7, 9), without examining any probable alteration in the receptor internalization rates, it is difficult to conclude which pathway of receptor cycle has been affected by the overexpression of these proteins. As with other GPCRs, the interaction between the \(\delta\)-opioid receptor and \(\beta\)-arrestin is the key for the agonist-induced rapid desensitization. Interaction with \(\beta\)-arrestin leads to both uncoupling from G protein and internalization of the receptor. Agonist-induced rapid desensitization of the \(\delta\)-opioid receptor can be abolished completely only when both of these processes are blocked. Whether the involvement of these two cellular events is required for the agonist-induced rapid desensitization of the \(\delta\)-opioid receptor in neuronal cell lines or in neurons remains to be demonstrated.

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