FEBS Letters 415 (1997) 200–205 FEBS 19272

Agonist-induced signaling and trafficking of the μ-opioid receptor: role of serine and threonine residues in the third cytoplasmic loop and C-terminal domain

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Received 15 July 1997; revised version received 25 August 1997

Abstract The human μ-opioid receptor and a mutant form, μS/T[i3+Cter]A, in which all Ser and Thr residues from the third cytoplasmic loop and C-terminal domain were changed to Ala, were studied after expression in CHO-K1 cells. Although the mutant receptors had similar affinities for agonists and EC_{50} values for inhibition of adenylyl cyclase as compared to wild-type receptors, the E_{max} were almost 2-fold decreased, suggesting a role of the mutated residues in G-protein coupling. After chronic morphine or etorphine, the EC_{50} values of the agonists were about 5-fold increased at both receptors but the E_{max} values were not altered; upon agonist withdrawal forskolin-stimulated cAMP levels were increased to almost 200% of control levels. Sequestration and rapid down-regulation of the μ-opioid receptor were induced by DAGO and etorphine but not morphine. In contrast, the μS/T[i3+Cter]A receptor was not sequestered and was up-regulated (150–380%) after treatment with agonists. The results indicate that the Ser and Thr residues in the third cytoplasmic loop and C-terminus of the μ-opioid receptor are not involved in the limited desensitization or in the adenylyl cyclase superactivation promoted by agonists but that their integrity and/or their phosphorylation is required in the intricate and coordinately regulated pathways involved in receptor signaling and trafficking.

Key words: Mu-opioid receptor; G -protein; Phosphorylation; Desensitization; Receptor trafficking

1. Introduction

Three main classes of integral membrane receptors, termed μ, δ and κ, mediate the pharmacological actions of opioids by coupling to regulatory G-proteins and modulating K^+ and Ca^{2+} channels, adenylyl cyclase, and phospholipase C activities. Morphine and alkaloid derivatives acting through μ receptors remain the most powerful drugs for the treatment of pain but their administration is limited by undesirable side effects, including tolerance and dependence. Tolerance is characterized by a decreased responsiveness to opioids upon continuous or repeated exposure to the drugs. Dependence is expressed upon withdrawal of the opioid, or administration of an antagonist, by physiological symptoms often opposite to the acute effects of the drug. In the long term, tolerance and dependence involve profound modifications of neuronal activity which are certainly triggered by adaptive changes of opioid receptor properties.

For several G-protein-coupled receptors exemplified by the β-adrenergic receptors, attenuated cellular responsiveness to agonists, also known as desensitization, has been correlated to alterations of receptor coupling to G-proteins and to internalization and down-regulation of receptors. Phosphorylation by various kinases (PKA, PKC and GRK) of serine and threonine residues located in the third cytoplasmic loop and carboxy-terminal tail of the receptors is believed to be at the basis of the desensitization process which also involves intracellular effectors such as β-arrestins and other regulators of G-proteins signaling [1].

Early studies using whole animals and model cell lines have failed to reveal consistent links between modifications of opioid responsiveness and receptor properties. The recent cloning of opioid receptor cDNAs has made it possible to better explore the possible adaptive changes of receptor function after sustained exposure to agonists. It has thus been suggested that agonist-stimulated phosphorylation of the μ-opioid receptor could lead to its constitutive activation rather than to desensitization and that this would be responsible for the development of narcotic tolerance and dependence [2,3]. In contrast, phosphorylation of δ- and κ-opioid receptors has been suggested to induce desensitization of both receptors [4,5].

In the present study, we expressed in CHO-K1 cells the wild-type μ-opioid receptor and a mutant form termed μS/T[i3+Cter]A where serine and threonine residues in the third intracellular loop and carboxy-terminal tail have been replaced by alanine, to investigate the possible implication of these regions in the adaptive processes of μ-opioid receptor signaling.

2. Materials and methods

2.1. Construction and expression of wild-type and mutant μ-opioid receptors

Unique restriction sites were introduced by silent oligonucleotide-directed mutagenesis into the human μ-opioid receptor cDNA [6] to facilitate the construction of mutant receptors. The sequences encoding L261-K262 and T281-R282 in the third intracellular loop (i3), and that encoding E393-A397 in the C-terminus of the receptor were respectively modified to create *Afl*II, *Mlu*I and *Sfi*I restriction sites. The μS/T[i3+Cter]A mutant in which all Ser and Thr residues from i3 (3 Ser and 1 Thr) and the carboxy-terminal tail (4 Ser and 7 Thr) were substituted by Ala was obtained by replacing the *Afl*II to *Mlu*I, *Ppu*10I to *Eco*RI and *Eco*RI to *Sfi*I fragments with synthetic oligonucleotides containing the desired mutations and restrictions sites

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PII S0014-5793(97)01124-1

convenient for screening purposes. Constructs were verified by sense and anti-sense sequencing throughout the modified regions.

The coding regions of receptors were subcloned into the pRc/CMV expression vector (Invitrogen, San Diego, CA, USA) and transfected into CHO-K1 cells (CCL-61, ATCC, Rockville, MD) by the calcium phosphate procedure. G418-resistant clones (400 μg/ml) were individually grown and subcloned by limiting dilution before screening for μ receptor expression using binding of 1 nM [^{3}H]diprenorphine to whole cells.

2.2. Receptor binding assays

Membrane preparation and receptor binding assays were performed as described [6]. For saturation experiments, 10–100 μg membrane proteins were incubated in 500 μl of binding buffer with 13 concentrations of [^{3}H]diprenorphine ranging from 0.1 to 3 nM. Inhibition of [^{3}H]diprenorphine (0.4 nM) binding was performed with concentrations of competitors ranging from 10^{-11} to 10^{-5} M. Non-specific binding was determined in the presence of 1 μM diprenorphine. Following a 1 h incubation period at 25°C, free ligand was removed by filtration onto Whatman GF/B filters and bound radioactivity was measured. Data were analyzed with the Inplot 4 program (Graphpad Software Inc., San Diego, CA, USA). Results are presented as the mean ± S.E.M. of three independent experiments performed in duplicate.

Down-regulation and sequestration of μ-opioid receptors were studied on whole cells plated into a 24-well dishes (2×10^5 cells/well) and allowed to recover overnight. Fresh medium alone or containing agonists (10 μM DAGO, 10 μM morphine, or 1 μM etorphine) was then added. Ligands were removed by sequential 30 min washes at 4°C: four washes in Ham's F12 supplemented with 10% fetal bovine serum and two washes in PBS containing 0.2% bovine serum albumin. Cells were then incubated for 2 h on ice in the latter buffer supplemented with 2.5 nM [^{3}H]diprenorphine. After three rapid washes with PBS/BSA 0.2%, cells were lysed using a solution of 0.2 N NaOH and 0.5% SDS, and lysates were harvested and counted. Intracellular binding sites and non-specific binding were defined as occurring in the presence of 10 μM DAGO and 1 μM diprenorphine, respectively.

2.3. cAMP accumulation assays

Cells were seeded in 12-well culture plates (10^5 cells/well) and allowed to attach for 24 h. Cells were then incubated four 4 h at 37°C with 0.6 μCi/well of [^{3}H]adenine (23 Ci/mmol, Amersham). Adenylyl cyclase activity was stimulated by the addition of 10 μM forskolin (FS) in 0.25 ml HEPES buffered Krebs-Ringer saline (KRH) containing phosphodiesterase inhibitors (0.1 mM IBMX and 0.1 mM Ro 20-1724) in the presence or absence of varying concentrations of agonists ranging from 10^{-11} to 10^{-5} M. After incubation for 10 min at 37°C, 25 μl of 2.2 N HCl was added and cAMP levels were measured [7]. For chronic treatments, opioid agonists were added to cultures for 4 h together with [^{3}H]adenine. Four rapid washes with 0.5 ml of KRH were performed to achieve withdrawal of opioid agonists and cAMP accumulation assays were performed as above. For efficient removal of the slowly dissociating etorphine, cells were rinsed twice with KRH buffer containing 100 μM naloxone, incubated for 10 min at 37°C in the same medium and rinsed four times with KRH alone. Data are expressed as the percentage of FS-stimulated cAMP accumulation in untreated cells.

3. Results

3.1. Pharmacological properties of the μ and μS/T[i3+Cter]A opioid receptors

Saturation binding experiments revealed that both the μ and the μS/T[i3+Cter]A opioid receptors expressed in CHO-K1 cells had comparable affinities for [^{3}H]diprenorphine (0.10 ± 0.01 nM vs. 0.29 ± 0.02 nM), which agreed with those measured in mammalian tissues or transfected cells. Expression levels were 3.5 ± 0.5 and 0.6 ± 0.1 pmol/mg of membrane proteins for the wild-type and mutant receptors, respectively.

Competition of [^{3}H]diprenorphine binding by DAGO, morphine, and etorphine showed that affinities of agonists were identical for μ and μS/T[i3+Cter]A receptors when inhibition curves were fitted to a one-site model (wild-type vs. mutant; 1.3 ± 0.2 nM vs. 1.7 ± 0.2 nM for DAGO and 3.3 ± 0.3 nM vs. 5.3 ± 0.5 nM for morphine). For the μ receptor however, the DAGO and morphine curves were best fitted to a two-site model (n_{Hill} = 0.62 ± 0.03 and 0.59 ± 0.03, respectively). In the presence of 100 μM Gpp(NH)p the ratio of high affinity sites decreased from 50 to 30% for DAGO and from 43% to undetectable for morphine (Table 1). For these agonists, the mutant receptor presented a single affinity state similar to the low affinity state of the wild-type receptor, which was not influenced by the presence of Gpp(NH)p. Surprisingly, both proteins presented a single and comparable affinity for etorphine.

For μ and μS/T[i3+Cter]A receptors, modulation by agonists of FS-stimulated cAMP accumulation produced dose-dependent inhibition curves (not shown). The rank order of potency of agonists (etorphine ≫ DAGO > morphine) and EC_{50} values were similar for the two receptors but maximal inhibition levels were decreased for the μS/T[i3+Cter]A mutant (Table 2).

Table 1
Inhibition constants of agonist binding to μ and μS/T[i3+Cter]A opioid receptors expressed in CHO-K1 cells

Receptor	Agonist	K_i (nM)		R_H (%)
		K_{iH}	K_{iL}	
μ	DAGO	0.26 ± 0.05	7.0 ± 0.3	50 ± 3
	+Gpp(NH)p[a]	0.20 ± 0.01	5.7 ± 0.2	29 ± 1
	Morphine	0.40 ± 0.05	10.4 ± 0.5	43 ± 1
	+Gpp(NH)p	ND[b]	7.1 ± 0.3	ND
	Etorphine	0.23 ± 0.07		
μS/T[i3+Cter]A	DAGO	1.7 ± 0.2		
	+Gpp(NH)p	2.3 ± 0.2		
	Morphine	5.3 ± 0.5		
	+Gpp(NH)p	6.4 ± 1.8		
	Etorphine	0.36 ± 0.07		

Membranes from CHO cells were incubated with 0.4 nM [^{3}H]diprenorphine in the presence of varying concentrations of the indicated agonists. K_{iH} and K_{iL} refer to high and low affinity agonist binding sites of competition curves fitted to a two-site model. R_H represents the percentage of sites in the high affinity state for agonists. Results are the mean ± S.E.M. of three or four independent experiments performed in duplicate.
[a]Binding in the presence of 100 μM Gpp(NH)p.
[b]ND, not detectable.

Table 2
Inhibition of forskolin-stimulated cAMP accumulation in control and agonist-exposed CHO cells expressing the μ and μS/T[i3+Cter]A opioid receptors

Receptor	Agonist	Control		Agonist exposure (4 h)	
		EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
μ	DAGO	11 ± 1	84 ± 3	25 ± 5	86 ± 2
	Morphine	65 ± 4	76 ± 4	244 ± 24**	81 ± 3
	Etorphine	0.31 ± 0.01	93 ± 1	ND[a]	11 ± 5
	Etorphine[b]	0.66 ± 0.11	92 ± 3	4.7 ± 1.0*	87 ± 5
μS/T[i3+Cter]A	DAGO	14 ± 2	53 ± 3	23 ± 3	42 ± 1
	Morphine	48 ± 4	48 ± 6	200 ± 32*	59 ± 7
	Etorphine	1.3 ± 0.4	60 ± 5	ND[a]	3 ± 6
	Etorphine[b]	4.2 ± 2.0	61 ± 4	6.8 ± 2.3	62 ± 3

Cells were cultured for 4 h in the absence of agonists (control) or exposed to 10 μM DAGO, 10 μM morphine or 1 μM etorphine. cAMP assays were then performed in the presence of 10 μM FS and varying concentrations of the corresponding agonist. The resulting curves were used to calculate agonist half-effective concentrations (EC_{50}) and maximal inhibition levels of FS-stimulated cAMP accumulation (E_{max}). Data represent the mean ± S.E.M. of 3–6 independent experiments performed in triplicate or quadruplicate.
[a]ND, not determined.
[b]Values obtained after washing cells in the presence of 10^{-4} M naloxone (see text for details).
*$P < 0.05$, **$P < 0.01$, compared to untreated cells.

3.2. *Effects on adenylyl cyclase activity of chronic exposure of μ and μS/T[i3+Cter]A receptors to agonists*

Chronic exposure (4 h) to DAGO (10 μM) or morphine (10 μM) of μ and μS/T[i3+Cter]A receptors followed by restimulation by the same agonist yielded E_{max} values for inhibition of FS-stimulated adenylyl cyclase which were not affected as compared to untreated cells (Table 2). A slight decrease (4-fold) of morphine EC_{50} values was, however, observed for both receptors following morphine treatment. No modification of DAGO EC_{50} occurred after DAGO treatment.

Chronic activation by DAGO or morphine of wild-type followed by withdrawal of agonists led, as observed in other systems [8,9], to an increased (180% of control cells) FS-stimulated cAMP production (Fig. 1). A smaller but significant adenylyl cyclase overshoot was obtained with the μS/T[i3+Cter]A receptor exposed to DAGO (125%) or to morphine (145%). Inhibition of cAMP accumulation upon acute or chronic morphine, and adenylyl cyclase superactivation after sustained exposure to morphine were totally suppressed by pertussis toxin treatment (not shown), indicating that Gi/Go proteins were involved in these processes [9].

As in HEK-293 cells [10], responsiveness to etorphine of CHO-K1 cells expressing the μ-opioid receptor was abolished after a 4 h pretreatment with 1 μM etorphine; the same effect was obtained for the μS/T[i3+Cter]A receptor (Table 2). Neither of the two receptors displayed the adenylyl cyclase overshoot seen with DAGO and morphine (Fig. 1). On the contrary, the FS-induced cAMP accumulation was reduced to about 50% of that of untreated cells. However, upon addition of 10 μM naloxone together with FS in the adenylyl cyclase assay, cAMP concentrations returned to the level of untreated cells (Fig. 1). Such an effect of naloxone may reflect continuous activation of receptors by etorphine which indeed slowly dissociates from brain opioid binding sites [11].

Since naloxone may accelerate the dissociation of etorphine from opioid receptors [11], this antagonist was included during the washing procedure (see Section 2) to verify this possibility. Under these conditions, E_{max} values of etorphine at

Table 3
Regulation of cellular density and plasma membrane expression of μ and μS/T[i3+Cter]A opioid receptors

	[^{3}H]DPN binding site			
	μ		μS/T[i3+Cter]A	
	total (% of control)	DAGO inaccessible (% of total)	total (% of control)	DAGO inaccessible (% of total)
30 min exposure				
none	100	20 ± 1	100	23 ± 3
DAGO	58 ± 4**	31 ± 1**	103 ± 9	17 ± 1*
Morphine	94 ± 6	12 ± 2**	105 ± 6	11 ± 3**
Etorphine	75 ± 5**	58 ± 11*	106 ± 10	21 ± 3
24 h exposure				
none	100	18 ± 2	100	18 ± 6
DAGO	55 ± 3**	14 ± 1	155 ± 10**	10 ± 2
Morphine	90 ± 5	12 ± 2	376 ± 15**	4 ± 1
Etorphine	42 ± 3**	33 ± 7	208 ± 19**	14 ± 3

Binding of [^{3}H]diprenorphine was performed on intact cells exposed as indicated to 10 μM DAGO, 10 μM morphine or 1 μM etorphine. Total binding was defined as that displaceable by 1 μM diprenorphine and is expressed as the percentage of that occurring in untreated cells. DAGO inaccessible sites were those remaining in the presence of 10 μM DAGO. Results are the mean ± S.E.M. of 3–6 independent experiments performed in duplicate.
*$P < 0.05$, **$P < 0.01$, compared to untreated cells.

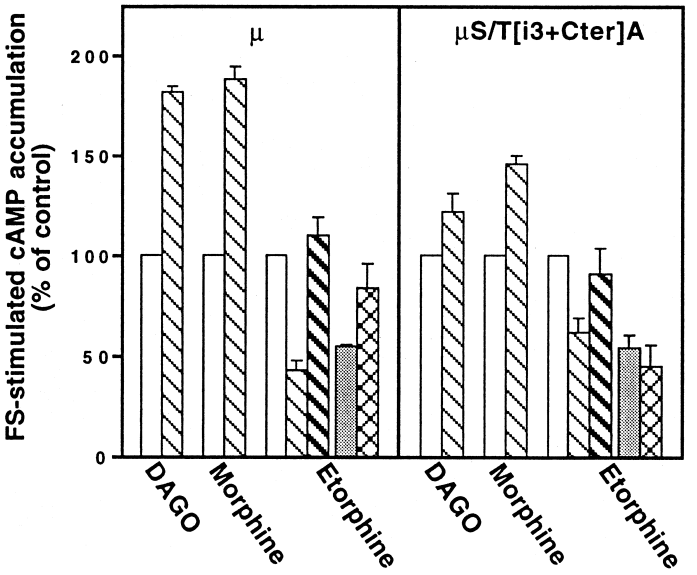

Fig. 1. Adenylyl cyclase superactivation following chronic exposure to agonists of CHO cells expressing μ or μS/T[i3+Cter]A opioid receptors. Cells were cultured for 4 h in the absence or presence of 10 μM DAGO, or 10 μM morphine, or 1 μM etorphine and rinsed four times with KRH. Results represent the percentage, as compared to control cells (white bars), of FS-stimulated cAMP accumulation in agonist-treated cells (hatched bars). Results obtained after chronic etorphine when 10 μM naloxone was added with FS in the cAMP assay are shown (bold-hatched bars). Since the lack of rebound effect after etorphine treatment may reflect receptor occupancy by etorphine, untreated cells (gray bars) or cells exposed to etorphine (cross-hatched bars) were washed in the presence of naloxone (see text for details) before measurement of FS-stimulated cAMP production.

each receptor were not altered by etorphine treatment (Table 2). A diminution of etorphine EC_{50} value was, however, found for the wild-type μ receptor and to a lesser extent for the μS/T[i3+Cter]A mutant. Control cells expressing μ or μS/T[i3+Cter]A receptors washed under these conditions displayed a 50% decrease in FS-stimulated cAMP levels (Fig. 1) which has also been observed in SH-SY-5Y cells [2]. Compared to these control cells, a significant superactivation of adenylyl cyclase (150–160%) was unmasked for the μ receptor but did not occur consistently for the μS/T[i3+Cter]A mutant (Fig. 1).

3.3. *Effects of chronic exposure to agonists of μ and μS/T[i3+Cter]A receptors on receptor density and sequestration*

After exposure to agonists, both surface and intracellular receptors were labelled using saturating doses (2.5 nM) of the lipophilic ligand [^{3}H]diprenorphine. Sequestered binding sites were defined as those occurring in the presence of 10 μM DAGO, a polar peptide which only blocked cell surface receptors [3]. In untreated cells, the ratio of sequestered to total binding sites (20%) was identical for the wild-type and the μS/T[i3+Cter]A receptors (Table 3). As reported for μ- and δ-opioid receptors [3,8,12–16], sequestration and/or internalization of the wild-type μ-opioid receptor was rapidly (30 min) induced upon activation by DAGO and etorphine. Surprisingly, μ receptors were also down-regulated after such a short exposition period to DAGO or etorphine. This is markedly distinct from the more slowly developing process (several hours) occurring for β-adrenergic receptors but has already been described for the μ-opioid receptor [14]. After longer periods (24 h) in the presence of DAGO or etorphine, the total number of binding sites was still reduced by about 50% but the ratio of sequestered sites returned to control values (Table 3). Neither sequestration nor down-regulation was observed with morphine which rather seemed to induce a slight diminution of DAGO inaccessible sites.

In contrast, no sequestration or down-regulation was observed with the μS/T[i3+Cter]A receptor which was instead up-regulated after 24 h of treatment with either agonist (Table 3). Moreover, the ratio of sequestered mutant receptors seemed to be decreased upon stimulation by morphine and this was accompanied by a much higher extent of receptor up-regulation as compared to DAGO and etorphine.

4. Discussion

4.1. *Ser and Thr residues in the third cytoplasmic loop and C-terminus are involved in coupling of the μ-opioid receptor to G-proteins*

Wild-type and μS/T[i3+Cter]A receptors had similar apparent affinities for the antagonist [^{3}H]diprenorphine and for the agonists DAGO, morphine and etorphine. The occurrence in the mutant receptor of a single affinity state for agonists, insensitive to Gpp(NH)p, did not reflect uncoupling from G-proteins since both receptors inhibited FS-induced cAMP accumulation with identical EC_{50} values. Moreover, in the wild-type μ receptor K_is at the low affinity state for agonists were in the same range as EC_{50} values, suggesting that even in this receptor the low affinity state for agonists substantially contributed to inhibition of adenylyl cyclase. As observed here for the wild-type and mutant μ receptors, others have reported on the existence of muscarinic [17] and 5-hydroxytryptamine [18,19] receptor agonists with a single affinity binding site insensitive to GTP analogs. As further supported in several systems by the existence of partial agonists with high binding affinity, this suggests that agonist affinity and efficacy are not directly related. Coupling of receptors to slow GDP-

GTP exchanging Go and Gq proteins, which mediate opioid as well as muscarinic and serotoninergic signals, may explain the lack of effect of Gpp(NH)p on agonist affinity.

The μS/T[i3+Cter]A receptor still had the ability to undergo isomerization to active conformations but the effects exerted by agonists on this process and/or on interactions of active conformations with G-proteins were certainly altered since the maximal inhibitory effects on adenylyl cyclase activity were lower for mutant than for wild-type receptors. Opioid receptors which are highly promiscuous in terms of coupling to G-proteins certainly exist under several conformations of different affinity for individual G-protein isoforms. The lower potency of the μS/T[i3+Cter]A receptor to inhibit adenylyl cyclase and the occurrence of a single affinity site for agonists may thus suggest that the order of affinity of the mutant receptor for the G-proteins normally interacting with μ receptors was modified, either because the mutated Ser and Thr residues directly interact with specific G-proteins or because they stabilize particular agonist-bound receptor conformations.

4.2. *Long-term exposure to agonists of μ-opioid receptors expressed in CHO-K1 cells does not induce receptor desensitization but promotes adenylyl cyclase superactivation*

Both the μ and μS/T[i3+Cter]A receptors chronically treated with DAGO, morphine or etorphine were able to modulate adenylyl cyclase activity upon stimulation by the corresponding agonist in an essentially similar fashion as before treatment. The decreased overshoot obtained with the μS/T[i3+Cter]A mutant may reflect the lower potency of the agonists at this receptor since adenylyl cyclase superactivation is mediated by βγ subunits of G-proteins and is thus proportional to the degree of receptor stimulation [20]. Thus, the limited desensitization observed for morphine and etorphine, and the adenylyl cyclase superactivation certainly did not involve receptor phosphorylation in the third cytoplasmic loop or in the carboxy-terminal tail. The phosphorylation sites present in the first and second cytoplasmic loops may, however, be implicated in such processes. Alternatively, this may be due to coupling of the activated receptors to different G-protein isoforms.

The present results on the long-term effects of agonists on the μ-opioid receptor expressed in CHO-K1 cells agree with those of Avidor-Reiss et al. [9] but differ from others in the same cell line [14] or in 7315c pituitary [12] and neuro$_2$ cells [8], where decreased EC_{50} values for morphine and DAGO were observed concomitantly with diminished maximal potencies. As for other receptors [21,22], such variations might be explained by tissue specificity of the desensitization process. Nevertheless, our results contradict those of Blake and collaborators [10] and suggest that the apparent desensitization and lack of rebound effect observed after chronic etorphine are best explained by receptor occupancy by this slowly dissociating agonist.

Adaptive changes in μ-opioid receptor responsiveness may also depend on experimental conditions since we and Avidor-Reiss et al. used whole cells whereas others used membrane fractions [12,14]. It is possible that one of the many proteins (GRKs, arrestins, phosducins, recoverins) which modulate signaling by G-proteins and coupled receptors [1] may be silenced in some cell types but that this necessitates an intact cellular machinery and/or particular experimental conditions. For example, the myristoylated brain membrane protein neurocalcin inhibits phosphorylation and desensitization of rhodopsin [23] but requires the presence of Ca^{2+} for proper interactions with tubulin and rhodopsin kinase [23].

4.3. *Ser and Thr residues in the third cytoplasmic loop and C-terminus are involved in agonist-regulated trafficking of the μ-opioid receptor*

Our results on μ-opioid receptor sequestration and down-regulation suggest a direct relationship between these two processes. Indeed, sequestration of wild-type μ-receptors occurred rapidly and concomitant with down-regulation upon exposure to DAGO and etorphine. Moreover, sequestration of μ receptors was not stimulated by morphine [3,15] but rather reduced, and this correlated with an absence of down-regulation. Furthermore, the ratio of sequestered μS/T[i3+Cter]A receptors was much lower upon morphine treatment, as compared to DAGO and etorphine, and was paralleled by a higher up-regulation of receptor number.

A recent model for trafficking of the β_2-adrenergic receptor postulates that receptors are dynamically exchanged between the plasma membrane and endosomes and from there are either recycled to the cell surface or directed towards lysosomes and degraded [24]. Agonists may modulate the cellular density and the membrane ratio of receptors by individually affecting each of the steps involved in trafficking: (i) sequestration into endosomes, (ii) recycling to the membrane or (iii) internalization into lysosomes. The agonist-bound β_2-adrenergic receptor is rapidly sequestered and predominantly recycled to the membrane, since receptor desensitization decreases G-protein coupling and agonist binding, two properties which are required for down-regulation (reviewed in [25]).

The absence of desensitization of the μ-opioid receptor expressed in CHO cells may thus favor the lysosome pathway resulting in rapid down-regulation. The receptor conformation(s) involved in the sequestration/recycling/degradation pathways could be largely disfavored in the μS/T[i3+Cter]A mutant, resulting in a degradation rate substantially lower than that of synthesis, eventually leading to receptor up-regulation. This may occur as a consequence of a limited receptor supply to the lysosomal compartment, arising either from an impaired ability of the μS/T[i3+Cter]A receptor to sequester into endosomes or from an increased rate of recycling due to the unphosphorylated state of this receptor. This latter possibility is suggested by studies on the β_2-adrenergic receptor demonstrating that receptors are dephosphorylated before being recycled to the plasma [25]. The unique property of morphine suggests that upon binding to μ or μS/T[i3+Cter]A receptors, this agonist may promote a receptor form of lower propensity to sequestration and/or degradation.

Previous studies on G-protein-coupled receptors, including the δ-opioid receptor [16], have demonstrated a role for Ser- and Thr-rich domains in the internalization/down-regulation processes [25]. From these and our studies, it remains unclear whether the role of these Ser and Thr residues relies on their chemical characteristics or on their phosphorylation. The present observations suggest that the Ser and Thr residues of the third cytoplasmic and C-terminal domains of the μ receptor may be implicated in receptor/G-protein interaction and/or in the conformational flexibility of the intracellular domains. Mutations such as those studied here in the μS/

T[i3+Cter]A receptor may thus affect one of the many steps involved in the intricate and coordinately regulated pathways of receptor signaling and trafficking.

Acknowledgements: This work was supported by grants from the Centre National de la Recherche Scientifique (ATIPE No. 1), the Université Paul Sabatier (Toulouse III), the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche (94.V.0244), the Conseil Régional de la Région Midi-Pyrénées (9307913 and 9407526), the Association pour la Recherche sur le Cancer (DOL94/6998) and the Fondation pour la Recherche Médicale.

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