Inverse Agonism and Neutral Antagonism at Wild-Type and Constitutively Active Mutant Delta Opioid Receptors


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ABSTRACT

The delta opioid receptor modulates nociceptive and emotional behaviors. This receptor has been shown to exhibit measurable spontaneous activity. Progress in understanding the biological relevance of this activity has been slow, partly due to limited characterization of compounds with intrinsic negative activity. Here, we have used constitutively active mutant (CAM) delta receptors in two different functional assays, guanosine 5′-O-(3-thio)triphosphate binding and a reporter gene assay, to test potential inverse agonism of 15 delta opioid compounds, originally described as antagonists. These include the classical antagonists naloxone, naltrexone, 7-benzylidene-naltrexone, and naltiben, a new set of naltrexolide derivatives, H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic[CH2N]Cha-Phe-OH [TICP(Ψ)], as well as three 2′,6′-dimethyltrosyne-1,2,3,4-tetrahydroquinoline-3-carboxylate (Dmt-Tic) peptides. A reference agonist, SNC 80 [(+)-4-[(αR)-α-(2S,5R)-4-Allyl-2,5-dimethyl-1-piperaziny]-3-methoxybenzyl]-N,N-diethylbenzamid, and inverse agonist, ICI 174864 (N,N-diallyl-Tyr-Alb-Alb-Phe-Leu), were also included. In a screen using wild-type and CAM M262T delta receptors, naltrex- one (NTI) and close derivatives were mostly inactive, and TIPP behaved as an agonist, whereas Dmt-Tic-OH and N,N(CH3)2-Dmt-Tic-NH2 showed inverse agonism. The two latter compounds showed negative activity across 27 CAM receptors, suggesting that this activity was independent from the activation mechanism. These two compounds also exhibited nanomolar potencies in dose-response experiments performed on wild-type, M262T, Y308H, and C328R CAM receptors. TICP(Ψ) exhibited strong inverse agonism at the Y308H receptor. We conclude that the stable N,N(CH3)2-Dmt-Tic-NH2 compound represents a useful tool to explore the spontaneous activity of delta receptors, and NTI and novel derivatives behave as neutral antagonists.

Evidence that a number of G protein coupled receptors (GPCRs) show spontaneous activity has accumulated in the past years, and the existence of high basal activity has been proposed for more than 60 GPCRs (Seifert and Wenzel-Seifert, 2002). Key tools to investigate receptor basal signaling are ligands that block this activity, the so-called inverse agonists (Milligan, 2003). In contrast to neutral antagonists, which compete with agonists but are devoid of biological activity per se, inverse agonists are endowed with intrinsic negative activity (Strange, 2002). Differential activity of inverse agonists and neutral antagonists was reported in recombinant systems and was also evidenced in physiological situations (de Ligt et al., 2000).

The concept of functional inverse agonism was pioneered with delta opioid receptors, which belong to the opioid receptor family comprising also μ, κ, and opioid receptor-like receptors (Kieffer, 1997). The finding that ICI 174864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) (Cotton et al., 1984), originally considered as delta antagonist, was able to decrease basal GTPase activity in NG108–15 neuroblastoma cells, whereas other antagonists had no effect in this functional assay, was the first demonstration of negative intrinsic activity at an endogenously expressed GPCR (Costa and Herz, 1989). The existence of delta receptor spontaneous activity was further confirmed using different functional assays in cell lines expressing either the endogenous (Szekeres and Traynor, 1997) or the recombinant delta receptor expressed in HEK 293 (Chiu et al., 1996; Labarre et al., 2000; Zaki et al., 2001), CHO (Hosohata et al., 1999), rat-1 fibroblast (Mullaney et al., 1996; Merkouris et al., 1997), GH3 (Liu and Prather, 2002), or C6 glioma cells (Neilan et al., 1999). From all these studies, ICI 174864 appeared to show reliable and potent negative activity and was taken as the reference molecule for delta inverse agonism. ICI 174864, however, is a peptidic ligand that shows low potency and selectivity at a wide range of delta compounds using a set of several CAM delta receptors. Our data show that the highly delta selective ligand N,N(CH3)2-Dmt-Tic-NH2 is most potent and efficacious at all the tested mutant receptors and seems to be a better inverse agonist than the reference ICI 174864 compound. TICP(Ψ) also showed inverse agonism, mainly at one mutant receptor. NTI derivatives were otherwise inactive, leaving NTI and closely related analogs as neutral antagonists.

Materials and Methods

Opioid Ligands. [3H]Diprenorphine (50 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Commercial delta opioid compounds were as follows: SNC 80, ICI 174864, and NLX (all from Sigma/RBI, Natick, MA), NTB, NTI, and BNTX (Tocris Cookson Inc., Ellisville, MO), and TIPP, where Tic is 1,2,3,4-tetrahydrossoquinoline (Phoenix Pharmaceuticals, Belmont, CA). The delta compound TICP(Ψ) was prepared as described by Schiller et al. (1996). Dmt-Tic molecules were synthesized as reported by Salvadori et al. (1995). The naltirindole derivatives were synthesized as described by Schutz et al. (2002).

Expression of Wild-Type and Mutant Receptors. For opioid ligand and [35S]GTP-S binding assays, the wild-type (WT) human delta receptor, the CAM M262T and the CAM C328R human delta receptors (Decaillot et al., 2003) were stably expressed in HEK 293 cells (American Type Culture Collection, Manassas, VA). Cells were transfected at about 70% confluence with WT or mutated delta receptor-expressing vectors (pcDNA3, 8 μg/10-cm dish) using the Jet-PEI reagent (Polytransfection, Strasbourg, France) according to the manufacturer’s protocol. Forty-eight hours after transfection, the culture medium, Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and gentamycin (Invitrogen), was replaced by fresh medium containing the selecting agent G418 (Invitrogen) at a 0.5 mg/ml concentration. Cells were grown at 37°C in the presence of 5% CO2. Resistant colonies appeared after 2 weeks of G418 application. Cellular clones were amplified and screened for receptor expression by [3H]diprenorphine binding on whole cells (0.75 and 1.5 nM, 2 x 105 cells/well). Clones with highest expression levels were amplified for further experiments. The Y308H mutant receptor was expressed transiently in HEK 293 cells. Transfection was performed in Dulbecco’s modified Eagle’s medium with 10% FCS with antibiotics, and cells were harvested after 48 h. For the selective alkaloid phosphatase (SEAP) assay, all WT and CAM receptors were expressed transiently in HEK 293 cells as previously described (Befort et al., 2001) with a slightly modified procedure. Briefly, HEK 293 cells were plated in 96-well plates (Biocoat; Falcon, Owley, UK) at a density of 35,000 cells/well on the day before transfection. Cells were cotransfected at about 70% confluency with a reporter gene pCRE-SEAP (1 μg/well; BD Biosciences Clontech, Palo Alto, CA) and WT or mutated delta receptor plasmid DNA (0.15 μg/well) using Jet-PEI reagent. Thirty-two hours after transfection, cells were serum-starved overnight and subjected to the SEAP test.

Ligand Binding Assay. HEK 293 cells expressing WT and CAM receptors stably or transiently were harvested for membrane preparation according to the described procedure (Befort et al., 2001). Membranes were resuspended in 50 mM Tris HCl, pH 7.4, and 0.32 M sucrose buffer, aliquoted, and stored at −80°C until use. Protein concentration was assayed by the Bradford method (Bio-Rad, Hercules, CA). For saturation experiments, 5 μg of membrane proteins were...
diluted in 50 mM Tris HCl, pH 7.4, in a final volume of 250 µl and incubated with variable concentrations (0.02–3 nM) of \([3H]\)diprenorphine for 1 h at 25°C. Nonspecific binding was determined in the presence of 10 µM naloxone. For competition studies, 5 µg of membrane proteins was incubated with 0.7 (C328R), 1 (WT and M262T), or 7 (Y308H) nM \([3H]\) diprenorphine, in the presence of variable concentrations (1 pM to 100 nM) of competing opioid ligand for 1h at 25°C. Membranes were washed with cold 50 mM Tris-HCl, pH 7.4, buffer by filtration through 0.1% polyethylenimine-presoaked microplate filters (Unifilter GF/B) using a Filtermate Harvester (PerkinElmer Life and Analytical Sciences). Microplates were dried, then wells were covered by 40 µl of scintillation cocktail (PerkinElmer Life and Analytical Sciences) and counted in a Microplate Scintillation/Luminescence Counter (Canberra Industries, Meriden, CT) using the TopCount-NXT software. Assays were performed in triplicates for saturation experiments and in duplicates for competition studies.

**K_d** and **K_i** values of opioid ligands were calculated using the Prism software (GraphPad Software Inc., San Diego, CA).

**[^35S]GTPγS Binding Assay.** Opioid ligand-stimulated \[^35S\]GTPγS binding was performed using the same membranes as
above. $[^{35}S]$GTPγS binding was carried out essentially as described previously (Befort et al., 1999). Briefly, 10 μg of membrane proteins was incubated for 1 h at 25°C without (basal) or with opioid ligands (3 pM to 10 μM) in the assay buffer containing 50 mM Hepes, pH 7.6, 5 mM MgCl$_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% bovine serum albumin, 30 μM GDP, and 0.2 nM $[^{35}S]$GTPγS. For background determination, 10 μM nonradioactive GTPγS was used. Incubation mixture was filtered as above (ligand binding section) except that microplate filters were presoaked in water. Plates were dried, and radioactivity was counted as above. Assays were performed in triplicates. EC$_{50}$ values of the various opioid ligands were determined with the Prism software using nonlinear regression and one-site competition. For each experiment, ligand-stimulated $[^{35}S]$GTPγS binding values were normalized to the basal value (absence of ligand) defined as 100%.

**SEAP Assay.** Cells were cotransfected using receptor and pCRIER-SEAP expression vectors as described above. Thirty-two hours after transfection, cells were serum-starved (0.1%, instead of 10% FCS) overnight. Forty-eight hours after transfection, cells were stimulated with forskolin (FK) alone (10 μM) or with forskolin and the opioid ligand to be tested. Treatments with all ligands were performed in the presence of 0.1% FCS for 5 to 6 h. Aliquots of culture medium supernatants (15 μl) were transferred into black 96-well plate, diluted into 150 mM NaCl and 40 mM Tris-Cl, pH 7.2, buffer and heat-inactivated at 65°C for 20 min. The enzymatic reaction was performed in the presence of assay buffer (666 mM diethanolamine, 3.3 mM MgCl$_2$, and 6.6 mM L-homoarginine, pH 9.8), enhancer solution (Tropics, Bedford, MA), and 0.4 mM SEAP chemiluminescent substrate (Tropics) in a final volume of 150 μl. Luminescence was quantified 15 min after substrate addition (TopCount-NXT, Canberra Industries). To compare data from different experiments for each compound and to allow comparison across mutant receptors, the SEAP signals obtained in the presence of FK and opioid compounds were normalized relative to signal obtained in the presence of FK alone, defined as 100%. A prototypic agonist (SNC 80) and the reference inverse agonist (ICI 174864) were included in each experiment. Dose response experiments were performed with agonist or inverse agonist concentrations ranging from (3 pM to 100 μM). Assays were performed in triplicate, and EC$_{50}$ values were calculated using the Prism software as above.

**Statistics.** In opioid ligand screening experiments (Fig. 2), one-way analysis of variance with protected least significant difference Fisher posteriori test was used to evaluate significant effects of the compounds ($p < 0.05$).

**Results**

### Screening for Inverse Agonism at WT and M262T Mutant Delta Receptors

In our previous search for CAM delta receptors, we have identified 30 point-mutated receptors with high spontaneous activity (Décaillot et al., 2003). The mutant M262T exhibited highest responsivity to ICI 174864 in the reporter gene assay and was, therefore, chosen as a screening tool in this study. Also, because the mutation is located on the cytoplasmic face of the sixth transmembrane domain and is, therefore, distant from the binding site, we reasoned that ligand binding should be preserved for most compounds under study. First, we established stable cell lines expressing the WT or M262T receptors. Receptor expression levels were determined by Scatchard analysis of $[^{3}H]$diprenorphine binding. $B_{max}$ values were 4.38 ± 0.91 pmol/mg for the WT receptor ($n = 2$) and 1.57 ± 0.35 pmol/mg for the M262T receptor ($n = 2$). Basal $[^{35}S]$GTPγS binding at the mutant receptor was consistently higher than WT (142.1 ± 7.8% of WT, $n = 3$) despite a 2.7-fold lower expression level, confirming that M262T is a CAM receptor (Décaillot et al., 2003). $[^{3}H]$Diprenorphine affinity was unchanged at this mutant receptor (Table 1). To examine whether the M262T mutation alters binding affinities of the delta ligands under study, we determined $K_i$ values for each compound (Table 1). Affinity changes never exceeded 4-fold, and with the exception of ICI 174864, all compounds exhibited similar nanomolar affinity for WT and M262T mutant receptors. Functional activity of all the compounds was, therefore, tested.

We first used the classical $[^{35}S]$GTPγS binding assay that measures receptor-mediated G protein activation occurring at an early stage of the signaling cascade. In this assay, $[^{35}S]$GTPγS binding is increased in the presence of agonist and decreased in the presence of inverse agonist. We treated membrane preparations from the two stable cell lines (WT and M262T) with each delta opioid ligand, and results are shown in Fig. 2 (top panel). The agonist SNC 80 increased basal $[^{35}S]$GTPγS binding about 2-fold at both WT and mutant receptors. As expected, ICI 174864, the reference delta inverse agonist, decreased the basal signal. In this set of experiments, the negative activity of ICI 174864 was low at WT and significant at the M262T mutant receptor, consistent with the expected higher efficacy of inverse agonists at CAM receptors (Décaillot et al., 2003). No significant modification of basal $[^{35}S]$GTPγS binding could be detected for NLX, NTI, BNTX, NTB, and TIPP at WT and M262T receptors. Naltrindole derivatives (HS compounds) showed no inverse agonist activity at any receptor and even showed a trend toward agonism at the WT receptor. Finally the compounds TICP(Ψ), Dmt-Tic-OH, and $N,N(CH_2)_2$-Dmt-Tic-NH$_2$ showed significant inverse agonism at preparations expressing the M262T receptor. The three compounds also showed detectable, although not always significant, negative activity at the WT receptor. Altogether, therefore, the three latter compounds appeared as the most efficacious inverse agonists in this assay.

To further examine bioactivity of the delta opioid compounds, we used the more sensitive high-throughput SEAP reporter gene assay, where the endpoint measurement is performed downstream from G protein activation along the cAMP pathway. In this assay, both a cAMP-responsive reporter gene and the receptor are transiently coexpressed in HEK 293 cells. FK stimulation directly activates the reporter gene, and the amount of gene product (SEAP) is measured using a chemiluminescent substrate. Typically, an opioid agonist will decrease the FK-stimulated reporter gene activity (SEAP activity), whereas an inverse agonist will enhance this activity (Décaillot et al., 2003). Data are shown in Fig. 2 (bottom panel). The delta opioid agonist, SNC 80, decreased the SEAP signal at levels corresponding to 50% (WT) and 70% (M262T) of levels observed in the absence of agonist. ICI 174864 increased the SEAP signal at cells expressing both WT (120% above basal) and M262T (130% above basal) receptors. As in the $[^{35}S]$GTPγS binding assay, this activity better detected at the mutant receptor. NLX, BNTX, Dmt-Tic-OH, and $N,N(CH_2)_2$-Dmt-Tic-NH$_2$ exhibited significant inverse agonist activity at the M262T receptor, whereas only $N,N(CH_2)_2$-Dmt-Tic-NH$_2$ markedly increased SEAP activity at the WT receptor. TICP(Ψ), which showed inverse agonism in the $[^{35}S]$GTPγS assay, was inactive in the SEAP assay. Conversely, TIPP, which was inactive in $[^{35}S]$GTPγS assay,
exhibited strong agonism in the SEAP test at both receptors. Finally, no significant effect of HS compounds could be observed at WT and M262T receptors, except for a slight agonist effect for HS-595. NTI and NTB were inactive at both receptors as well.

Taken together, screening data from both functional as-

Fig. 2. Screening for inverse agonist activity. Fifteen delta compounds were tested on WT (left) and M262T mutant (right) receptors. Delta opioids include, from left to right: the reference agonist SNC 80 (left black bars), the reference inverse agonist ICI 174864 (right black bars), commercial alkaloids (NLX, NTI, BNTX, and NTB) and peptide (TIPP), the TIPP analog TICP(Ψ), three Dmt-Tic compounds, and six novel NTI derivatives (gray bars). Top panel, ligand-induced [\textsuperscript{35}S]GTP\textsubscript{S} binding. Ligand concentrations were 10 μM (except SNC 80 at 1 μM). [\textsuperscript{35}S]GTP\textsubscript{S} binding activities were normalized to basal activity (without ligand) defined as 100% (horizontal line). Data are means ± S.E.M. from three to five experiments performed in triplicate and using at least two independent membrane preparations. Bottom panel, ligand-induced SEAP activation. Ligand concentrations were at 1 μM (except ICI 174864 at 10 μM) in the presence of 10 μM FK. SEAP activities were normalized to signal obtained with FK alone, defined as 100% and indicated by a horizontal line. Data are means ± S.E.M. from three to nine independent transfection experiments performed in triplicate. Activities of the tested compounds were compared with basal values using the Student’s t test. One star, p < 0.05; two stars, p < 0.01; three stars, p < 0.001 (one-way analysis of variance).
TABLE 1
Binding affinities of delta compounds to WT and mutant delta receptors

<table>
<thead>
<tr>
<th>Compounds</th>
<th>WT</th>
<th>M262T</th>
<th>Y308H</th>
<th>C328R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$</td>
<td>$n$</td>
<td>$K_i$</td>
<td>$n$</td>
</tr>
<tr>
<td>$[^3]H$Diprenorphine ($K_d$)</td>
<td>0.39 ± 0.06</td>
<td>2</td>
<td>0.72 ± 0.21</td>
<td>2</td>
</tr>
<tr>
<td>SNC 80</td>
<td>9.41 ± 3.07</td>
<td>3</td>
<td>9.62 ± 2.59</td>
<td>3</td>
</tr>
<tr>
<td>ICI 174864</td>
<td>434 ± 61</td>
<td>4</td>
<td>1308 ± 247</td>
<td>3</td>
</tr>
<tr>
<td>NLX</td>
<td>66.20 ± 0.71</td>
<td>2</td>
<td>59.11 ± 2.92</td>
<td>2</td>
</tr>
<tr>
<td>NTI</td>
<td>0.30 ± 0.15</td>
<td>3</td>
<td>0.12 ± 0.02</td>
<td>3</td>
</tr>
<tr>
<td>NTB</td>
<td>0.10 ± 0.02</td>
<td>3</td>
<td>0.23 ± 0.04</td>
<td>3</td>
</tr>
<tr>
<td>TIPP</td>
<td>2.90 ± 0.66</td>
<td>3</td>
<td>7.03 ± 3.05</td>
<td>3</td>
</tr>
<tr>
<td>TICP(Ψ)</td>
<td>4.62 ± 0.64</td>
<td>3</td>
<td>3.91 ± 0.27</td>
<td>3</td>
</tr>
<tr>
<td>H-Dmt-Tic-OH</td>
<td>13.13 ± 1.71</td>
<td>3</td>
<td>5.20 ± 1.85</td>
<td>3</td>
</tr>
<tr>
<td>H-Dmt-Tic-NH$_2$</td>
<td>9.41 ± 1.13</td>
<td>3</td>
<td>9.43 ± 1.16</td>
<td>3</td>
</tr>
<tr>
<td>N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$</td>
<td>2.50 ± 0.76</td>
<td>3</td>
<td>1.81 ± 0.66</td>
<td>3</td>
</tr>
<tr>
<td>HS-378</td>
<td>0.40 ± 0.01</td>
<td>2</td>
<td>0.90 ± 0.03</td>
<td>3</td>
</tr>
<tr>
<td>HS-414</td>
<td>2.10 ± 0.72</td>
<td>3</td>
<td>4.13 ± 1.04</td>
<td>3</td>
</tr>
<tr>
<td>HS-464</td>
<td>0.11 ± 0.03</td>
<td>3</td>
<td>0.40 ± 0.09</td>
<td>3</td>
</tr>
<tr>
<td>HS-510A</td>
<td>0.12 ± 0.03</td>
<td>3</td>
<td>0.41 ± 0.15</td>
<td>3</td>
</tr>
<tr>
<td>HS-531</td>
<td>0.42 ± 0.08</td>
<td>2</td>
<td>0.80 ± 0.09</td>
<td>2</td>
</tr>
<tr>
<td>HS-595</td>
<td>0.22 ± 0.01</td>
<td>2</td>
<td>0.41 ± 0.05</td>
<td>2</td>
</tr>
</tbody>
</table>

says and from both WT and M262T CAM receptors led to the following conclusions: TIPP and HS-595 show either no activity or agonist activity; NTI, NTB, and the other HS derivatives show no significant activity in any test, despite their high affinity for the receptors; NLX and BNTX show inverse agonism in one test and for one receptor only; and as for ICI 174864, Dmt-Tic-OH, N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$, and TICP(Ψ) behave as inverse agonists. The latter compounds were, therefore, studied further.

**Inverse Agonist Activity of TICP(Ψ), Dmt-Tic-OH, and N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$ across a Series of CAM Receptors.** We examined the negative functional activity of ICI 174864, TICP(Ψ), Dmt-Tic-OH, and N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$ across a series of CAM receptors. We performed a control experiment where empty HEK 293 cells were transfected with the empty pcDNA3 plasmid and subjected to these compounds ($10^{-5}$ M) in both [3H]GTP$\gamma$S binding and SEAP assays. All compounds were inactive (data not shown), indicating that their activity is mediated by delta receptors.

We investigated whether ICI 174864, TICP(Ψ), Dmt-Tic-OH, and N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$, which show inverse agonist activity at WT and M262T mutant receptors, would also inhibit the high spontaneous activity of other CAM delta receptors independently from the position of the mutation. To test the activity of the compounds at a large set of mutant receptors, we used the SEAP assay that does not require membrane preparations. We expressed 27 CAM delta receptors from our previous study (Décaillot et al., 2003) and measured the modulation of SEAP responses after TICP(Ψ), Dmt-Tic-OH, and N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$ stimulation. Each compound was used at a saturating concentration (1 $\mu$M) and systematically compared with ICI 174864 (10 $\mu$M). Maximal effects are shown in Fig. 3. As in our first experiment (Fig. 2), the most active inverse agonist was N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$. This compound enhanced the FK-induced SEAP signal at all other CAMs (Fig. 3, top panel). For the stronger effects, the rank of order was M262T > T213S = T134A = Y308H > C328R = E323K = V283A. This effect was more important than the effect of ICI 174864 at most CAM receptors, suggesting that N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$ is a more efficacious inverse agonist. As for this compound, the closely related compound Dmt-Tic-OH behaved as a delta inverse agonist at most CAM receptors (Fig. 3, middle panel). For Dmt-Tic-OH, highest levels of activation were obtained at mutant receptors T134A, T213S, V283A, and C328R. When data from this ligand were directly compared with those of N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$, the overall efficacy appeared lower (data not shown). Also, the inverse agonist activity of Dmt-Tic-OH was lower than the reference compound ICI 174864 for mutants D21G, N169S, W274R, and E323K, which was not the case for N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$. Dmt-Tic-OH, therefore, seemed to be a less efficient inverse agonist than N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$. TICP(Ψ) was inactive at most mutants (data not shown). The compound showed detectable activity at mutant receptors M262T and C328R. Interestingly, the inverse agonist effect of TICP(Ψ) was remarkably active at the Y308H receptor, reaching 220% of the FK-stimulated signal (Fig. 3, bottom panel). This was the highest value observed across all mutants and for all ligands.

Together, these data show that ICI 174864, TICP(Ψ), Dmt-Tic-OH, and N,N(CH$_3$)$_2$-Dmt-Tic-NH$_2$ all exhibit inverse agonism at several CAM delta receptors. This observation confirms that these compounds are endowed with robust negative intrinsic activity, which favors the inactive receptor conformation, even though distinct activation mechanisms are likely to be involved in the different mutants (Décaillot et al., 2003). The four compounds show variable efficacies depending on the CAM receptors. At this stage, comparing the compounds is difficult because these differences could be due either to distinct intrinsic functional activities or because mutations alter binding affinities of the compounds to variable extents. Therefore, we focused our attention on the three CAM receptors M262T, Y308H, and C328R, which exhibited the highest basal activities (see our previous work, Décaillot et al., 2003), and where inverse agonists showed best activities in this study (Fig. 3).
Dose Responses to Dmt-Tic Compounds at WT, M262T, Y308H, and C328R Mutant Receptors. To fully characterize agonism and inverse agonism of the most active delta compounds, we first determined their binding affinities at the different receptors (Table 1). We have shown above that opioid binding at M262T delta receptor is comparable with WT receptor. Similarly, $K_i$ values were unchanged at the C328R mutant, consistent with the notion that M262 and C328 residues are located on the cytoplasmic face of the receptor and unlikely involved in ligand binding (Décaillot et al., 2003). In contrast, binding affinities were substantially decreased at the Y308H receptor. This was expected because this residue is centrally located within the ligand binding pocket and known to influence opioid affinity (Befort et al., 1996, 1999). Affinities to Y308H, however, remained measurable, and dose responses were also performed for this mutant.

Further, we determined potencies and efficacies using the two functional assays at the selected receptors. For $[^{35}S]$GTPyS binding assays, we used stable expression for WT ($B_{\text{max}}$, 4.38 ± 0.91 pmol/mg protein; $n = 2$), M262T ($B_{\text{max}}$, 1.57 ± 0.35 pmol/mg protein; $n = 2$), C328R mutants ($B_{\text{max}}$, 5 pmol/mg protein) and transient expression for Y308H receptor ($B_{\text{max}}$, 7.99 ± 1.26 pmol/mg protein; $n = 2$). For the SEAP assay, receptors were expressed transiently, as before.
The agonist SNC 80 increased \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding and decreased the SEAP response at WT and M262T receptors, as in our previous experiments. In the \(^{35}\text{S}\)GTP\(_{\gamma}\)S assay, potencies (EC\(_{50}\)) of SNC 80 were similar at WT (EC\(_{50}\), 2.79 ± 0.85 nM; \(n = 3\)) and M262T (EC\(_{50}\), 0.48 ± 0.14 nM; \(n = 4\)) receptors. In the SEAP assay also, EC\(_{50}\) values were comparable at WT (EC\(_{50}\), 0.17 ± 0.06 nM; \(n = 5\)) and M262T mutant (EC\(_{50}\), 0.16 ± 0.01 nM; \(n = 4\)) receptors. In the \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding assay, maximal responses (\(E_{max}\)) were similar for the two receptors (WT, \(E_{max}\), 187.8 ± 6%; M262T, \(E_{max}\), 174.5 ± 5.9% from basal binding). In the SEAP assay, the agonist efficacy was higher at the WT receptor (\(E_{max}\), 48.6 ± 4.3% from basal) compared with the M262T receptor (\(E_{max}\), 71.0 ± 4% from basal). At the Y308H mutant, SNC 80 activity was low in the SEAP assay (\(E_{max}\), 85.3 ± 1.1% from basal; EC\(_{50}\), 100.41 ± 62.21 nM; \(n = 3\)) and undetectable in the \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding test, partly due to the fact that the Y308H mutation strongly impairs SNC 80 binding (46-fold decrease in affinity, see Table 1) and that \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding is less sensitive compared with SEAP assay. SNC 80 was also inactive on mutant C328R (data not shown), although in this case, SNC 80 binding affinity was not impaired (see Table 1). From our previous data (Décaillot et al., 2003), it is likely that the strong spontaneous activity of both Y308H

Fig. 4. Dose responses for inverse agonist activity of \(N,N\)-(CH\(_3\))\(_2\)-Dmt-Tic-NH\(_2\). Dose response curves were established on WT, M262T, Y308H, and C328R CAM receptors (top to bottom) using \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding (left) or the SEAP reporter gene assay (right). Responses to the reference agonist and inverse agonist (SNC 80 and ICI 174864) are displayed together with responses to \(N,N\)-(CH\(_3\))\(_2\)-Dmt-Tic-NH\(_2\). For purpose of clarity only one representative experiment is show in each experimental condition. EC\(_{50}\) and efficacy values from several experiments are indicated in Table 2 and in text. In the \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding assay, membranes were incubated with the ligand at concentrations ranging from 3.16 pM to 100 μM. \(^{35}\text{S}\)GTP\(_{\gamma}\)S binding activities were normalized to basal activity (absence of ligand), defined as 100%. For the SEAP assay, transfected cells were incubated for 5 h with FK alone (10 μM) or with various concentrations of the ligand (3 pM to 100 μM). SEAP activity values were normalized to the FK signal (100%). SNC 80 was ineffective at the C328R mutant.
and C328R mutants limits further modulation by an agonist. Whether other delta opioid agonists are able to activate these mutant receptors remains to be tested. Representative dose responses for SNC 80 activity at WT, M262T, and Y308H mutants are shown in Fig. 4.

The reference inverse agonist ICI 174864 decreased $[^{35}S]$GTP-γ-S binding and increased SEAP responses at WT and mutant receptors (Figs. 4 and 5). In the $[^{35}S]$GTP-γ-S assay, ICI 174864 efficacies ($E_{\text{max}}$) were 76.9 ± 2.4% (WT, n = 2), 73.9 ± 2.4% (M262T, n = 4), 92.8 ± 2.2% (Y308H, n = 2), and 68.5 ± 2.6% (C328R, n = 2) of basal values. $E_{\text{max}}$ in the SEAP test were 147.5 ± 21.5% (WT, n = 3), 170.1 ± 9.5 (M262T, n = 5), 174.2 ± 12.7% (Y308H, n = 3), and 159.5 ± 21.8% (C328R, n = 3) of FK-stimulated signal. Potencies could not be determined accurately, reflecting the extremely low affinity of ICI 174864 compound toward WT and mutant delta receptors (>400 nM, Table 1) compared with the other delta compounds.

Potencies and efficacies of $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ are shown in Table 2. Inverse agonism of this compound was significant at the WT receptor in the two functional assays. Efficacies were consistently higher at CAM receptors compared with WT, reaching 63.8 ± 2.5% and 248.5 ± 16.7% of basal values in the $[^{35}S]$GTP-γ-S binding and SEAP assays, respectively (see Y308H receptor). Potencies paralleled affinities (Table 1) for all the receptors and were in the low nanomolar range. The inverse agonist activity of $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ is illustrated by representative dose-response curves displayed in Fig. 4, where functional activity of this compound is compared with those of the reference agonist SNC 80 and inverse agonist ICI 174864.

The closely related Dmt-Tic-OH compound showed similar properties (Table 2). Potencies and efficacies were comparable with those of $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ at the WT receptor but tended to be lower at mutant receptors, particularly in the SEAP assay. Together with our previous observation of an overall lower efficacy across the 27 mutant receptors (Fig. 3), it seemed that inverse agonism was slightly less robust for this compound. Adding to this, results from Dmt-Tic-OH were generally less reliable across experiments (data not shown), probably due to lower stability of the compound that forms a diketopiperazine (Capasso et al., 1995; Balboni et al., 1997) and subsequently loses activity (Balboni et al., 1997). $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ remains, therefore, the best candidate for future inverse agonist studies, particularly in physiological preparations.

Finally, we tested whether the inverse agonist activity of $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ could be reversed by a neutral antagonist. In a new set of experiments, we observed that SNC 80 (1 μM) and $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ (1 μM), respectively, decreased and increased the FK-induced SEAP signal at the M262T receptor (64 ± 11% and 193 ± 2%, n = 2), as earlier. Both these activities were inhibited in the presence of NTI (1 μM), with SEAP signal values close to values obtained with FK alone (102 ± 9% and 104 ± 2%, n = 2, respectively). Similar results were obtained for the WT receptor (data not shown).

**Dose Responses of TICP(Ψ) at WT, M262T, Y308H, and C328R Mutant Receptors.** Although inverse agonist activity was detected at saturating concentrations (Figs. 2 and 3), and although affinities at all the receptors were in the nanomolar range (Table 2), we could not obtain reproducible EC50 values in $[^{35}S]$GTP-γ-S binding dose-response experiments at WT, M262T, and C328R receptors (data not shown). However, $E_{\text{max}}$ values were consistent across experiments (WT, 79.1 ± 1.9%, n = 2; M262T, 77.3 ± 1.8%, n = 7; Y308H, 73.8 ± 3.6%, n = 3), demonstrating negative effect of this compound at these mutants. Interestingly, TICP(Ψ) was extremely active at the Y308H receptor (Fig. 5). In the $[^{35}S]$GTP-γ-S binding assay, TICP(Ψ) decreased the basal signal by 66.2 ± 5.6% (n = 5). In the SEAP assay, the enhancement of FK activation reached 227.7 ± 12.3% (n = 4) of basal level. Therefore, in both functional assays, maximal effects of TICP(Ψ) at this mutant were comparable with those obtained with $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ and corresponded to maximal changes that could be obtained under our experimental conditions. EC50 values were 20.7 ± 5.6 ($[^{35}S]$GTP-γ-S binding) and 17.4 ± 2.2 (SEAP) nM and were comparable with the $K_I$ value at this receptor (Table 1). In conclusion, TICP(Ψ) showed spectacular inverse agonism at the Y308H receptor and modest inverse agonism at the WT and other CAM receptors.

**Discussion**

Inverse agonism at delta opioid receptors was previously demonstrated in various cell lines expressing either native or wild-type recombinant receptor. Here, we have used a collection of mutant receptors with high basal activity as another approach to examine inverse agonist properties of delta compounds. We have examined delta ligands from distinct structural families, and our data show that $N,N'\text{(CH}_3\text{)}_2\text{-Dmt-Tic-NH}_2$ exhibits consistent and potent inverse agonism at WT and mutant receptors, TICP(Ψ) displays negative activity in a receptor-dependent fashion, and NTI and related derivatives are essentially neutral compounds.

An innovative aspect of this study is the use of CAM delta receptors to better assess inverse agonism of delta opioid compounds. Indeed, when the 15 compounds were screened for negative activity, stronger activities were detected at the M262T mutant compared with WT receptor. This demonstrates that, as was shown for other GPCRs (Rossier et al.,

![Fig. 5. Dose responses for inverse agonist activity of TICP(Ψ) at the Y308H receptor. TICP(Ψ) activity was measured in $[^{35}S]$GTP-γ-S binding experiment (left) and in the SEAP assay (right). Responses to the reference agonist and inverse agonist (SNC 80, ICI 174864) are also represented for comparison. $[^{35}S]$GTP-γ-S binding activities as well as SEAP responses were normalized to basal activity (absence of ligand = 100%). EC50 and efficacy values from four ($[^{35}S]$GTP-γ-S) and four (SEAP) independent experiments are indicated in Results.](image-url)
TABLE 2

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Potencies and efficacies of Dmt-Tic compounds to enhance the FK-stimulated SEAP signal and decrease basal [35S]GTPγS binding at WT and mutant delta receptors

EC50 and maximal activation values are calculated from dose-response curves for each ligand in both functional assays. Representative experiments for N,N(CH2)2-Dmt-Tic-NH2 are shown in Fig. 4. Maximal responses to the compounds were normalized to basal [35S]GTPγS binding or FK-stimulated SEAP response obtained in the absence of ligand, defined as 100%. Values are means ± S.E.M. from n separate experiments performed in triplicate.

1999; Behan and Chalmers, 2001), a CAM delta receptor is a better detector of inverse agonism than the WT receptor. Confirming this, inverse agonist efficacies of the various compounds were generally comparable at the WT receptor, whereas partial and full inverse agonism could be distinguished at the several CAM delta receptors, when full dose responses were performed (see Figs. 4 and 5).

The inverse agonist activity of ICI 174864 at the WT receptor appeared modest in our expression system, compared with previous studies that have used other cellular models (see Introduction and references therein). Replacing Na+ by K+ ions, for example, which successfully increased ICI 174864 activity in the case of excitatory cells such as NG108-15 (Costa and Herz, 1989; Szekeres and Traynor, 1997) or GH3 cells (Liu and Prather, 2002), was ineffective in our HEK 293 cells. Therefore, when HEK 293 cells are used as host cells, CAM receptors represent a clear advantage for the study of inverse agonism.

Another interesting finding was that the negative activity of the Dmt-Tic compounds was significant at many distinct CAM receptors, independently from the localization of the mutation. Evidence that different mutations may produce multiple active conformations has been suggested by us and others on the basis of signaling or trafficking studies (see Whistler et al., 2002 or Décaillot et al., 2003, among others). Our finding that Dmt-Tic compounds are active at all the tested CAM receptors, with mutations distributed throughout the receptor proteins, strongly suggests that these compounds are able to inhibit the several potentially distinct active conformations (Décaillot et al., 2003). Dmt-Tic molecules should, therefore, represent reliable and potent inverse agonists in distinct experimental contexts.

The three pseudopeptides from the Dmt-Tic family (Bryant et al., 2003) all showed negative activity in the [35S]GTPγS binding screening experiment. The reason why inverse agonism of Dmt-Tic-NH2 was not confirmed in the SEAP assay, despite a better sensitivity of this assay, remains open. Dmt-Tic-OH and N,N(CH2)2-Dmt-Tic-NH2 otherwise remained strong inverse agonists throughout the study, the latter exhibiting consistently slightly better activity. N,N(CH2)2-Dmt-Tic-NH2, therefore, appears as a full inverse agonist, with an efficacy comparable with that of ICI 174864 and with a much higher potency (nanomolar instead of micromolar). Labarre et al. (2000) compared functional activity of the same three Dmt-Tic compounds in a [35S]GTPγS binding study using a HEK 293 cell line stably expressing the WT delta receptor. They showed neutral antagonism, partial agonism, and full inverse agonism for Dmt-Tic-NH2, Dmt-Tic-OH, and N,N(CH2)2-Dmt-Tic-NH2, respectively. Both their data and our study, therefore, put forward N,N(CH2)2-Dmt-Tic-NH2 as the more active delta inverse opioid agonist. As many other Dmt-Tic opioids, this compound is delta selective (K delta, 0.31 ± 0.05 nM; K mu, 511.4 ± 74.6 nM; Salvadori et al., 1995). In addition, because the N-methylation augments hydrophobicity and prevents spontaneous cyclization (Bryant et al., 2003), N,N(CH2)2-Dmt-Tic-NH2 is considered a stable compound (Bryant et al., 2003). Altogether, therefore, N,N(CH2)2-Dmt-Tic-NH2 appears to be the best pharmacological tool to explore the spontaneous activity of delta receptors.

Peptides from the TIPP family showed unanticipated activities. TIPP was originally developed as a delta opioid antagonist (Schiller et al., 1992). Consistent with this observation, TIPP was inactive in our [35S]GTPγS binding assay. However, in more recent studies, Martin et al. (2001) reported that TIPP could show agonistic properties in several cell lines using cAMP measurements. In addition, the latter authors showed that, in GH3 cells, TIPP exhibited inverse agonist or agonist properties, depending on whether the end-point measurement was performed early (receptor binding) or late (cAMP response) along the signaling cascade (Martin et al., 2002). In line with these observations, we found agonistic properties for TIPP in the SEAP reporter gene assay that is sensitive to cAMP levels. Together with the study by Martin et al., therefore, our data suggest that TIPP may activate receptors to a small extend, and that the agonistic nature of TIPP is revealed after signal amplification only. This activity, however, may not be easily detectable since Pineyro et al. (2001) showed no TIPP-induced modifications of cAMP levels when the delta receptor was expressed in HEK 293 cells. Differences between the study of Pineyro et al. and our study may arise from distinct assay conditions. Whether TIPP behaves as agonist or inverse agonist under physiological conditions remains to be explored.

TICP(Ψ) was derived from TIPP by reduction of the peptide bond between Tic2 and Phe3 and saturation of the Phe3 aromatic ring, leading to a chemically stable pseudopeptide with high delta selectivity (K delta, 0.259 nM; K mu.1050 nM; Schiller et al., 1996). Like TIPP, TICP(Ψ) was reported to be
a potent antagonist (Schiller et al., 1999). In their study assessing functional activities of TIPP (see above), Pineyro et al. (2001) also examined TICP(Y) and reported that TICP(Y) acted as a full inverse agonist (similar to ICI 174864) for the cAMP response. In our study, TICP(Y) showed only modest negative activity at the WT delta receptor, although we used a similar expression system and a similar endpoint measurement. Clearly, the detection of negative activity is dependent on the exact assay conditions.

TICP(Y) nevertheless exhibited inverse agonism at the mutant Y308H receptor in our study. In fact, not only TICP(Y) but also the two Dmt-Tic compounds behaved as full inverse agonists at this receptor, whereas ICI 174864 activity was low. The Y308H receptor, therefore, seemed particularly responsive to peptides from the TIPP and Dmt-Tic families. The reason why TICP(Y) showed strong inverse agonism at this receptor and, more generally, why the pseudopeptides were highly active at the Y308H receptor remains unexplained. In contrast to mutations M262T and C328R, this mutation is located centrally within the binding pocket and drastically modifies opioid binding (Befort et al., 1996, 1999). Specific atomic interactions may take place between these inverse agonists and the signaling core of the receptor (Tm 3/Tm 6/Tm 7; Decaillot et al., 2003) that strongly favor the inactive receptor conformation. Three-dimensional modeling studies, in the future, may clarify specific structural features within the TICP(Y)-Y308H receptor complex.

Labarre et al. (2000) showed weak partial inverse agonism for the NTI derivative HS-378. Our data showed no activity for this compound, as well as for a set of five closely related HS compounds, at the CAM M262T receptor. We even observed weak partial agonism at the WT receptor for most of them. Together, it seems that NTI and NTI derivatives (HS compounds) are weakly active at the delta receptor, and this activity could be slightly negative (Labarre et al., 2000), neutral, or slightly positive (this study) depending on the experimental system. Compared with ICI 174864 and the two active Dmt-Tic compounds, these compounds could, therefore, be considered as neutral ligands.

In conclusion, we have investigated the functional activity of several peptide and nonpeptidic delta opioid compounds across delta receptors with variable levels of spontaneous activity. Our data indicate that the N,N(CH$_2$)$_3$Dmt-Tic-NH$_2$ compound represents a useful inverse agonist to modulate the spontaneous activity of delta receptors in recombinant and possibly physiological situations. Our data also show that TIPP peptides are endowed with intriguing properties that deserve further exploration in different experimental conditions. NTI derivatives remain essentially neutral in our experiments, and the most selective compounds (HS ligands) may serve as neutral antagonists in future experiments.

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References


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