Mutagenesis of the Mouse Delta Opioid Receptor Converts (–)-Buprenorphine from a Partial Agonist to an Antagonist

GEORGE BOT, ALLAN D. BLAKE, SHUIXING LI and TERRY REISINE
From the Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania
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ABSTRACT
An aspartic acid at residue 95 (Asp95) in the delta receptor has previously been shown to be critical for the binding affinity of selective delta agonists. To gain a better understanding of the functional consequence of agonist action at the delta receptor, the Asp95 residue was mutated to an asparagine (D95N) and opioids were tested for binding and functional activation of the wild-type and mutant delta receptors. Selective agonists such as [δ-Ser2,δ-Leu5]enkephalin-Thr6 (DSLET) and [δ-Ala2,δ-Leu5]enkephalin (DADLE) had greatly reduced affinity for the D95N mutant receptor but still inhibited cAMP accumulation, which indicated that the mutant receptor was still functionally coupled to adenylyl cyclase. Antagonist binding was not affected by the Asp95 mutation. Similarly, the partial agonist buprenorphine bound with equally high affinity to the D95N mutant and the wild-type delta receptor, which indicated that Asp95 is not essential for the binding affinity of this opioid. Buprenorphine did not affect cAMP accumulation in HEK 293 cells expressing the D95N mutant, and it blocked the ability of DSLET and bremazocine to inhibit cAMP accumulation via the D95N mutant, which indicated that buprenorphine acts as an antagonist at the D95N mutant. These findings confirm the essential role of Asp95 in the activation of the delta receptor by agonists and reveal a molecular basis of the unique property of buprenorphine.

Opoid analgesics are used extensively in the management of pain. However, a limitation to their effectiveness is the development of dependence, a condition for which an effective treatment is unavailable. Buprenorphine is a synthetic oripavine analgesic structurally related to the potent opioid agonist etorphine, and to diprenorphine, an antagonist. It exhibits potent analgesic and antinociceptive actions (Cowan, 1995; Lewis, 1995) with a limited capacity for producing physical dependence and is less reinforcing than other opiates (Nogues and Woods, 1995). Although used primarily in clinical pain management (Foley, 1993), buprenorphine is under development as a treatment for opioid dependence and causes a functional desensitization with regard to cAMP inhibition, but no receptor internalization, after pretreatment of the mouse mu receptor expressed in HEK 293 cells (Blake et al., 1997). The pharmacological profile of buprenorphine has been suggested to reflect a mu agonist at low doses and mu and kappa antagonists at high doses. Some authors have suggested that these mixed agonist/antagonist properties of buprenorphine contribute to its clinical effectiveness (Rothman et al., 1995; Dykstra and Nogues, 1995).

Currently, little is known about the functional consequence of buprenorphine on delta receptor function. Binding studies have shown that buprenorphine binds with high affinity to the delta receptor (Kong et al., 1993; Rothman et al., 1995); peripheral administration of buprenorphine up-regulated delta receptors in the forebrain region of the rat brain (Belcheva et al., 1993), which suggests an antagonistic action at the delta receptor for buprenorphine. Other studies, however, have reported inhibition of cAMP accumulation in COS cells expressing the cloned mouse delta opioid receptor by buprenorphine (Kong et al., 1993). These studies suggested that buprenorphine may also display an agonist/antagonist characteristic at the delta receptor.

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and have distinct binding determinants. Mutation of aspartic acid residue 95 (Asp$^{95}$) and 128 (Asp$^{128}$) of the delta receptor to an asparagine created mutant receptors, D95N and D128N, respectively, which exhibited reduced affinity for delta selective agonists such as DSEL and DPDPE but not for antagonists such as naltrindole, BNTX and NTB (Kong et al., 1993; Befort et al., 1996a). Hence the D95N and D128N mutants maintained between agonist and antagonist binding, and the Asp$^{95}$ and Asp$^{128}$ residues were essential for the binding of delta selective agonists.

In the present study we demonstrated that buprenorphine binding affinity to the delta receptor was not decreased by mutation of either Asp$^{95}$ or Asp$^{128}$ to an asparagine. However, most importantly, we showed that the Asp$^{95}$ mutation in the delta receptor converted the partial agonistic character of buprenorphine to a pure antagonist. We also showed that full delta selective agonists were much less potent in activating the D95N mutant than the wild-type delta receptor, which indicates that Asp$^{95}$ is essential for full-agonist action at the delta receptor.

Materials and Methods

Cell culture. HEK 293 cells were grown and maintained in minimal essential medium with Earle’s salts (Life Technologies, Inc., Grand Island, NY) containing 10% fetal calf serum, 100 units/ml penicillin and streptomycin sulfate in 10% CO$_2$ at 37°C. The mouse delta opioid receptor cDNA in pcDNA3 (Invitrogen, San Diego, CA) modified with the FLAG epitope (DYKDDDDK) at the amino terminus was a generous gift from Dr. Mark von Zastrow, University of California, San Francisco. The mouse delta opioid receptor, the D95N and D128N mutant cDNAs were stably transfected into HEK 293 cells by a modification of the calcium phosphate protocol (Chen et al., 1996). HEK 293 cell monolayers at approximately 70% confluence were transfected with 30 μg of plasmid. After an overnight incubation at 37°C, the medium was removed and the cells were treated with 5 ml of phosphate-buffered saline containing 10% glycerol for 10 min at room temperature. Cells were then washed twice with phosphate-buffered saline and incubated for 48 h at 37°C in growth medium. Stable transformants were selected in growth medium containing 1.0 mg/ml bacitracin and 0.5 μg/ml aprotinin and placed on ice. A cell pellet was prepared by centrifugation at 24,000 × g for 7 min at 4°C and was homogenized in the same buffer by a Polytron (Brinkmann Instruments, Westbury, NY) at setting 2.5, 30 sec. The cell homogenate was centrifuged at 48,000 × g for 20 min at 4°C, and the resulting cell pellet was homogenized and placed on ice for the binding assays. Binding assays were carried out at 25°C for 40 min in a final volume of 200 μl with 1 nM $[^{3}H]$naltrindole as radioligand and 1 μM naltrindole to define nonspecific binding. The binding reaction was terminated by the addition of ice-cold 50 mM Tris-HCl (pH 7.8) and rapid filtration over FP-100 Whatman GF/B glass fiber filters that were pretreated with 0.9% polyethyleneimine and 0.1% bovine serum albumin. The filters were rinsed with 12 ml of ice-cold buffer, and the bound radioactivity was determined by use of a liquid scintillation counter. Total binding and nonspecific binding for the wild-type delta receptor were typically 2560 ± 400 cpm and 380 ± 30 cpm, respectively, and for the D95N mutant 1520 ± 210 cpm and 230 ± 40 cpm, respectively.

cAMP accumulation studies. Stably transfected HEK 293 cells were subcultured in 12-well culture plates and allowed to recover for 72 h before the experiments. For agonist pretreatment studies, a 10-fold concentrated stock of agonist was diluted into growth medium and added to individual culture wells for the times indicated in the table and figure legends. The final concentration of all agonists used in regulation studies was 1 μM. After treatment, the medium was removed and replaced with 1 ml of growth medium containing 0.5 mM IBMX and the cells were incubated for 30 min at 37°C. The growth medium was then replaced with fresh medium containing 10 μM forskolin with or without agonist in the concentration range of 10$^{-12}$ to 10$^{-6}$ and the cells transferred to 37°C. After 5 min the medium was removed, 1.0 ml of 0.1 N HCl was added and the monolayers frozen at −20°C. For determination of the cAMP content of each well, the monolayers were thawed, placed on ice, sonicated, and the intracellular cAMP levels measured by radioimmunoassay (Amersham plc, Buckinghamshire, UK). Data obtained from the dose-response curves were analyzed by nonlinear regression analysis with GraphPad Prism 2 (GraphPad Software, Inc. San Diego, CA).

Results

To investigate buprenorphine regulation of the cloned delta opioid receptor, the wild-type cDNA and mutant form of the delta receptor that contained an aspartate to asparagine substitution at amino acids 95 (D95N) and 128 (D128N) were both stably expressed in HEK 293 cells. Pharmacological characterization of the stably transformed cells was carried out by radioligand binding and the functional inhibition of forskolin-stimulated cAMP accumulation, as described previously (Kong et al., 1993; Raynor et al., 1994; Befort et al., 1996a). Saturation binding with the delta selective radioligand, $[^{3}H]$naltrindole, demonstrated that the wild-type delta receptor was expressed in HEK 293 cells at the level of 9.4 ± 3.0 pmol mg$^{-1}$ of membrane protein ($B_{max}$) with a dissociation constant of ($K_{D}$) of 0.3 ± 0.06 nM (n = 3). Saturation analysis of $[^{3}H]$naltrindole binding revealed a $K_{D}$ of 1.3 ± 0.5 nM (n = 3) and $B_{max}$ of 59.0 ± 9.0 fmol mg$^{-1}$ protein for the D95N mutant and a $K_{D}$ of 1.5 ± 0.2 nM (n = 3) and $B_{max}$ of 236.0 ± 30.0 fmol mg$^{-1}$ protein for the D128N mutant. These results indicate that the mutant delta receptors were expressed at a lower density than the wild-type. No specific $[^{3}H]$naltrindole binding was detected in untransfected HEK 293 cells (data not shown).

A series of opioids were tested for their binding affinity to the wild-type and D95N mutant delta receptor (table 1). The analysis of competitive radioligand binding data with $[^{3}H]$-
altrindole showed that the expressed wild-type delta receptor had specific, high-affinity binding for the delta selective opiates DSLET, DPDPE, DADLE and SIOM as well as the nonselective ligands bremazocine and (+)-buprenorphine.

The binding affinities were similar to those previously reported in rat brain membranes (Rothman et al., 1995), in HEK 293 cells (Wang et al., 1995; Bot et al., 1997) and in other surrogate cell lines (Kong et al., 1993; Raynor et al., 1994; Befort et al., 1996a; Meng et al., 1996). Consistent with previous results (Kong et al., 1993), delta selective nonpeptide, SIOM, and peptide agonists, such as DSLET and DPDPE, exhibited reduced affinities for the D95N mutant, whereas bremazocine exhibited a small reduction (table 1). DSLET did not bind to the D95N mutant, whereas SIOM and DPDPE bound with affinities of approximately 0.5 μM. Similar to the results of Kong et al. (1993), the antagonist naltrindole displayed similar affinities at the wild-type and D95N mutant delta receptor (table 1). In agreement with the antagonist binding data, (+)-buprenorphine exhibited similar binding affinities at both receptors (table 1).

Studies on opioid receptors expressed in HEK 293 cells have shown that these receptors are coupled to the inhibition of adenyl cyclase and to G proteins of the Gi or Gs family (Arden et al., 1995; Tsu et al., 1995; Pei et al., 1995). The cloned delta receptor expressed in HEK 293 cells was functionally active and mediated agonist inhibition of forskolin-stimulated cAMP accumulation (fig. 1). The selective delta agonist DSLET and the nonselective agonists bremazocine and buprenorphine inhibited cAMP accumulation (table 1).

Their potencies are similar to previously published potencies for the delta selective and nonselective agonists acting at the delta receptor to inhibit cAMP accumulation in the mouse NG108–15 hybrid cells (Cai et al., 1996; Pei et al., 1995) and in delta receptor transfected CHO cell line (Evans et al., 1992; Law et al., 1994; Malatynska et al., 1996), COS-7 cell line (Kong et al., 1993) and HEK 293 cells (Keith et al., 1996; Bot et al., 1997). The potencies of most agonists to inhibit cAMP accumulation were greater than their binding affinities. We have no direct explanation for this difference but it is possible that this could be caused by the presence of spare receptors as suggested from the studies of Law et al. (1994).

The large spare-receptor pool may consist of high-affinity and G protein-coupled receptors only a small proportion of which needs to be stimulated to inhibit cAMP accumulation, whereas the binding studies detect both coupled and uncoupled low-affinity delta receptors. Hence the binding studies would detect a mixture of high- and low-affinity receptors. Although the agonist binding displacement studies with [3H]naltrindole conformed best to a one-site binding model, as determined by analysis of the best-curve fit of the dose-response curve, the Hill coefficients of the agonists buprenorphine (0.64 ± 0.02), DSLET (0.55 ± 0.02), SIOM (0.54 ± 0.03) and bremazocine (0.77 ± 0.03) to displace [3H]naltrindole in the wild-type delta receptor were significantly less than 1.0, which suggests the existence of binding sites with different affinities for these compounds. Hence cAMP inhibition may not necessarily be related to opioid occupancy in a linear manner. The extent of maximal inhibition of buprenorphine (maximal inhibition %, n) (66.3 ± 4.4, n = 3), morphine

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding Kᵢ (nm)</th>
<th>EC₅₀ (nm)</th>
<th>Maximal inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSLET</td>
<td>7.6 ± 2.4</td>
<td>0.03 ± 0.01¹</td>
<td>80.3 ± 2.4</td>
</tr>
<tr>
<td>DPDPE</td>
<td>34.0 ± 6.0</td>
<td>0.09 ± 0.01²</td>
<td>88.3 ± 1.0</td>
</tr>
<tr>
<td>DSLET</td>
<td>18.0 ± 6.0</td>
<td>0.03 ± 0.01</td>
<td>82.8 ± 4.0</td>
</tr>
<tr>
<td>SIOM</td>
<td>63.0 ± 5.0</td>
<td>2.2 ± 1.7</td>
<td>54.7 ± 7.2</td>
</tr>
<tr>
<td>Morphine</td>
<td>N.D.</td>
<td>38.0 ± 2.1</td>
<td>54.3 ± 4.9</td>
</tr>
<tr>
<td>Bremazocine</td>
<td>10.6 ± 3.0</td>
<td>0.57 ± 0.1</td>
<td>71.0 ± 4.5</td>
</tr>
<tr>
<td>(+)-Buprenorphine</td>
<td>2.4 ± 0.6</td>
<td>1.4 ± 1.3</td>
<td>66.3 ± 4.4</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>5.7 ± 1.5</td>
<td>6.6 ± 1.9</td>
<td>33.5 ± 6.0</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>0.41 ± 0.06</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Naloxone</td>
<td>486.0 ± 58.0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

¹ Data from Bot et al. (1997).
² P < .05 (Student’s t test, compared with wild-type); ³ P < .01; ⁴ P < .001; ND, not determined.
and 17.8 ± 2.7%, respectively.

To further investigate whether differences in agonist interaction with D95N mutant are caused by variations in agonist binding, a series of opiates were tested for stimulation of the same mutant receptor as described by Kong et al. (1993). Consistent with their reduced binding affinities reported here and in previous studies (Kong et al., 1993), the delta selective agonists DSLET, DADLE, DPDPE and SIOM (table 1) and the nonselective agonist bremazocine (table 1, fig. 3) were much less potent in inhibiting cAMP accumulation in cells expressing the D95N mutant. This was reflected in a rightward shift of the dose-response curve (fig. 3, table 1). The D95N mutant also was no longer able to be desensitized after 3 h pretreatment with DSLET and levorphanol, agonists which have previously been reported to desensitize the delta receptor expressed in HEK 293 cells (table 3) (Bot et al., 1997). The inability of agonists to desensitize the D95N mutant may be caused by the agonists being less effective in activating cellular processes normally involved in desensitizing the wild-type delta receptor.

Although (-)-buprenorphine, and to a lesser extent the structurally related ligand diprenorphine, inhibited cAMP accumulation in HEK 293 cells expressing the wild-type delta receptor (table 1), they were incapable of inhibiting cAMP accumulation via the D95N mutant despite binding to the receptor with subnanomolar affinity (table 1, fig. 4). The loss of effectiveness of buprenorphine is unlikely to be caused by its low efficacy because SIOM, which was less efficacious than buprenorphine in inhibiting cAMP accumulation in the wild-type delta receptor (table 1), was still able to inhibit cAMP accumulation via the D95N mutant. Likewise morphine, which has also been reported as being a partial agonist at the delta receptor (Bot et al., 1997), was also still able to inhibit cAMP accumulation but with a reduced efficacy in the D95N mutant (% maximal inhibition: 28.0 ± 2.5, n = 3, Student’s t test to wild-type, P < .05) compared with the wild-type delta receptor (54.3 ± 4.9, n = 3). The high affinity of buprenorphine for the D95N mutant and the lack of functionality suggests that buprenorphine was acting as an antagonist at the D95N mutant receptor. To test this possibility, buprenorphine was examined for its ability to block DSLET and bremazocine inhibition of cAMP accumulation. DSLET and bremazocine inhibited cAMP accumulation to the same maximal extent (table 1) and when added together, produced

### Table 2

Effects of naltrindole and buprenorphine on DSLET inhibition of forskolin-stimulated intracellular cAMP production for the cloned mouse delta opioid receptor (delta WT) and the D95N mutant stably expressed in HEK 293 cells.

<table>
<thead>
<tr>
<th>DSLET ± ligand</th>
<th>Delta WT</th>
<th></th>
<th>D95N mutant</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC\textsubscript{50}</td>
<td>% Maximal inhibition</td>
<td>EC\textsubscript{50}</td>
<td>% Maximal inhibition</td>
</tr>
<tr>
<td>DSLET (alone)</td>
<td>0.03 ± 0.01</td>
<td>77.5 ± 4.3</td>
<td>5.9 ± 1.4</td>
<td>41.7 ± 2.5</td>
</tr>
<tr>
<td>+Naltrindole</td>
<td>1.03 ± 0.4</td>
<td>28.5 ± 3.9\textsuperscript{a}</td>
<td>140 ± 39\textsuperscript{b}</td>
<td>18.5 ± 3.7\textsuperscript{c}</td>
</tr>
<tr>
<td>+Buprenorphine</td>
<td>0.57 ± 0.2\textsuperscript{a}</td>
<td>74.2 ± 4.5</td>
<td>43.8 ± 9.1\textsuperscript{a}</td>
<td>15.0 ± 3.8\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} P < .05 (Student’s t test, compared with DSLET alone); \textsuperscript{b} P < .01; \textsuperscript{c} P < .001.
are the mean expressed in HEK 293 cells. Intracellular cAMP accumulation was as-
duced the ability of DSLET to inhibit cAMP via Buprenorphine (1 m) manner to block the actions of DSLET and bremazocine.

dissociation rates, they may act in a semi-noncompetitive be ruled out that because both compounds may have slow is known to be a competitive antagonist. However, it can not
this is likely caused by the high concentrations of buprenor-
dole and buprenorphine reduced the maximal effectiveness of
antagonist naltrindole (tables 2 and 4, figs. 5 and 6). Both naltrin-
dole and buprenorphine reduced the maximal effectiveness of
agonists to inhibit cAMP accumulation via the D95N mutant. This is likely caused by the high concentrations of buprenor-
phine and naltrindole used in this study, because naltrindole is known to be a competitive antagonist. However, it can not
be ruled out that because both compounds may have slow dissociation rates, they may act in a semi-noncompetitive manner to block the actions of DSLET and bremazocine. Buprenorphine (1 μM) did not reduce the maximal ability of DSLET to inhibit cAMP accumulation via the wild-type delta
receptor (table 2, fig. 2), which suggests that its binding to the wild-type delta receptor was competitive and that its

### TABLE 3

<table>
<thead>
<tr>
<th>Receptor</th>
<th>No Pretreatment</th>
<th>Agonist Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ligand</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Delta WT</td>
<td>DSLET</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Levophanol</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>D95N</td>
<td>DSLET</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Levophanol</td>
<td>4.0 ± 2.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < .05 (Student’s t test, compared with untreated cells); *P < .01.
<sup>b</sup> Delta WT values taken from Bot et al. (1997).

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**Fig. 4.** (—)-Buprenorphine inhibition of cAMP accumulation for the wild-type delta receptor, the D95N and the D128N mutant receptors expressed in HEK 293 cells. Intracellular cAMP accumulation was assayed as described under "Materials and Methods." The data presented are the mean ± S.E. of three or more separate experiments, each performed in duplicate.

the same maximal inhibition of cAMP accumulation as DSLET did alone (not shown) and did not exhibit a potency less than each agonist individually, which suggests that both are full agonists at the wild-type delta receptor.

In contrast to its action at the wild-type delta receptor (table 1), buprenorphine did not inhibit cAMP accumulation via the D95N mutant. When added to increasing concentrations of DSLET (0.1–1000 nM), buprenorphine (1 μM) reduced the ability of DSLET to inhibit cAMP via the D95N mutant (table 2, fig. 5). A similar antagonism of the effects of the agonist bremazocine was found when buprenorphine was used (table 4, fig. 6). The ability of buprenorphine to block agonist inhibition of cAMP accumulation via the D95N mutant was similar to the actions of the classical delta antagonist naltrindole (tables 2 and 4, figs. 5 and 6). Both naltrindole and buprenorphine reduced the maximal effectiveness of agonists to inhibit cAMP accumulation via the D95N mutant. This is likely caused by the high concentrations of buprenorphine and naltrindole used in this study, because naltrindole is known to be a competitive antagonist. However, it can not be ruled out that because both compounds may have slow dissociation rates, they may act in a semi-noncompetitive manner to block the actions of DSLET and bremazocine. Buprenorphine (1 μM) did not reduce the maximal ability of DSLET to inhibit cAMP accumulation via the wild-type delta receptor (table 2, fig. 2), which suggests that its binding to the wild-type delta receptor was competitive and that its actions at the wild-type delta receptor are consistent with it being a partial agonist, which would be expected to have agonist property per se but would diminish the potency of full agonists when combined with them (Jasper and Insel, 1992).

In addition to aspartic acid 95, recent studies have shown that another conserved aspartic acid at residue 128 (Asp<sub>128</sub>) is also critical for high-affinity agonist binding to the delta receptor. Mutations of Asp<sub>128</sub> to asparagine (D128N) have an important role in ligand recognition (Befort et al., 1996b).

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### TABLE 4

<table>
<thead>
<tr>
<th>Bremazone ± ligand</th>
<th>Delta WT</th>
<th>Delta Opioid Receptor/Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Maximal inhibition</td>
</tr>
<tr>
<td>Bremazone (alone)</td>
<td>0.03 ± 0.01</td>
<td>77.5 ± 4.3</td>
</tr>
<tr>
<td>+Naltrindole</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>+Buprenorphine</td>
<td>11.3 ± 4.2</td>
<td>78.0 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < .05 (Student’s t test, compared with bremazocine alone); *P < .01. ND, not determined.

**Fig. 5.** Effects of naltrindole and (—)-buprenorphine on DSLET inhibition of cAMP accumulation for D95N mutant delta receptors. HEK 293 cell-monolayers, expressing the D95N mutant delta receptors were treated with growth medium containing DSLET in the concentration range 10<sup>−11</sup> to 10<sup>−6</sup> M, together with 10 μM forskolin and 1 μM of either naltrindole or buprenorphine for 5 min at 37°C, and then assayed for intracellular cAMP levels as described under "Materials and Methods." The data presented are the mean ± S.E. of three or more separate experiments, each performed in duplicate.
Furthermore, aromatic amino acid residues in the transmembrane spanning regions have also contributed to agonist binding to the delta receptor (Befort et al., 1996b). Our results indicate that the charged amino acid, Asp$^{95}$, is also involved in agonist binding, but more importantly, is critical for agonist activation and desensitization of the delta receptor.

Our previous results showed that mutation of Asp$^{95}$ to asparagine greatly reduced affinity of the delta receptor for selective agonists (Kong et al., 1993). In the present study we show that the potency and efficacy of delta selective agonists such as DPDPE, DSLET, DADLE and SIOM to activate the D95N mutant and hence to inhibit cAMP accumulation was reduced, even though the receptor was coupled to G proteins (Kong et al., 1993). However, more importantly, buprenorphine was unable to activate the D95N mutant expressed in HEK 293 cells, even though its affinity to bind to the mutant receptor was not reduced by the mutation. Buprenorphine acted as an antagonist at the D95N mutant blocking the effects of the agonists DSLET and bremazocine to inhibit cAMP accumulation. This is similar to the result of Molleyreau et al. (1997) who reported that a replacement of Gln$^{280}$ by His in TM6 of the human ORL1 opioid receptor-like receptor-G protein associations for DSLET and buprenorphine increased the binding affinity of lofentanil and etorphine but reduced their intrinsic activity to inhibit cAMP accumulation. Consequently, they no longer acted as pure agonists, as they do at the native ORL1 receptor, but exhibited clear antagonistic properties.

Behavioral studies in humans and nonhuman primates have suggested that buprenorphine is a mixed agonist/antagonist at opioid receptors (see the introduction). This was also suggested by our results in that its effectiveness in inhibiting cAMP accumulation via the wild-type delta receptor was less than that exhibited by the full agonists such as DSLET; it also reduced the potency of DSLET and bremazocine to inhibit cAMP accumulation without altering their maximal inhibitory capacity. This is consistent with the concept of partial agonism as proposed by Jasper and Insel (1992). It is conceivable that mutation of the Asp$^{95}$ residue removed the agonist component of buprenorphine and hence removed the ability of buprenorphine to activate intracellular G protein associated effector systems, while retaining the ability of the compound to bind to the delta receptor. In support of this idea, it has been suggested recently that opioids exhibit marked differences in efficacy and/or potency in the activation of different G proteins (Garzon et al., 1997). Different receptor-G protein associations for DSLET and buprenorphine may contribute to the presence and absence, respectively, of inhibition of cAMP accumulation in the D95N mutant.

Mutagenesis studies have indicated that antagonists do not bind to the same domains in delta opioid receptors as agonists (Kong et al., 1993; Befort et al., 1996a, b). This may explain why antagonists such as naltrindole and naloxone bound to the delta wild-type and the D95N mutant receptors with similar affinities whereas full agonists exhibited greatly reduced affinities at the D95N mutant. The similar affinities of buprenorphine for the wild-type and D95N mutant delta receptors suggests that buprenorphine may bind to the receptor in a manner similar to antagonists. In fact, (−)-buprenorphine has a chemical structure very similar to (−)-diprenorphine (fig. 7) which may provide a basis for its similar binding affinity at the wild-type and D95N mutant receptor.
agonist binding and activation of G-protein-linked receptors, including the delta receptor (Befort et al., 1996b). Mutation of Asp$^{128}$ to asparagine resulted in a receptor with greatly reduced affinity for DADLE and DPDPE as well as selective nonpeptide agonists. These findings showed that the Asp$^{128}$ of the delta receptor has an important role in ligand recognition. Consistent with the results of Befort et al. (1996a) that the D128N mutant had lower affinity for delta selective agonists, we have found that Asp$^{128}$ also influences agonist activation of the delta receptor. Selective agonists such as DADLE, DPDPE and DSLET where less potent at inhibiting cAMP accumulation in D128N mutant delta receptor expressing HEK 293 cells than cells expressing the wild-type delta receptor (Bot et al., 1997). Most of the selective agonists had similar maximal inhibitory effects on cAMP accumulation via the D128N and wild-type delta receptors, but exhibited decreased potency, which suggests that Asp$^{128}$ is critical for the affinity of the receptor for these agonists. In contrast, the effect of buprenorphine on the D128N mutant and wild type delta receptor were indistinguishable, which indicated that Asp$^{128}$ is not essential for this mixed agonist/antagonist to bind to and activate the delta receptor.

The results of this study suggest that full agonists and mixed agonist/antagonists may act via some common mechanisms to stimulate the delta receptor. The differences between these classes of compounds, however, may be in how they bind to the delta receptor and hence which intracellular-effector system(s) they activate. Because buprenorphine is an analog with limited abuse potential, identification of its ligand binding determinants may be useful in the development of novel opioids with limited abuse potential and limited tolerance after continued use.

Both (−)-buprenorphine and (−)-diprenorphine are N-cyclopentylmethyl-nordihydroorVinil derivatives originally prepared from thebaine-methyl vinyl ketone adducts in the search for analogues which may be superior to morphine but with fewer side effects. (For a full review of the chemistry of these compounds see Bentley, 1971.) They differ structurally only in the length of the alkyl chain group R at position C$_{19}$ with diprenorphine having a methyl whereas buprenorphine has a t-butyl substitution. Structure-activity relationships, based on rodent antinociceptive and morphine antagonism tests, established that increasing the length of the alkyl chain group R in the structure at position C$_{19}$ from methyl to t-butyl had little effect on mu antagonist selectivity but resulted in higher intrinsic activity (Lewis, 1974). Hence a primary alcohol (and diastereoisomeric methyl secondary alcohols) substitutes on C$_{19}$, as present in diprenorphine, convert the compound to an antagonist with low intrinsic activity, whereas propyl and butyl tertiary alcohol substitutes, as present in buprenorphine, impart a powerful analgesic character on the structure as determined in the rodent tail-pressure test (Lewis, 1974). Mutation of the Asp$^{95}$ residue may have removed the ability of the t-butyl and methyl groups to impart an agonistic character to buprenorphine and diprenorphine, respectively, perhaps by not allowing receptor association with intracellular-effector G protein systems which usually occur after agonist binding to the wild-type delta receptor. In support of this, recent reports have demonstrated dynamic changes in G protein association with the delta receptor after agonist binding (Law and Reisine, 1997). Furthermore, agonist activation has been reported to promote association of G$_{i/o_2}$ with the receptor, a G protein proposed to mediate the coupling of the delta receptor to adenylyl cyclase (McKenzie and Milligan, 1990). Hence this specific association of particular G proteins with the wild-type delta receptor may have been altered in the D95N mutant. This structural separation of inherent agonist/antagonist character by the D95N mutant was only evident for the N-cyclopentylmethyl-nordihydroorVinil derivatives diprenorphine and buprenorphine, and not for the other partial agonists SIOM and morphine, which still retained activity via the D95N mutant, which suggests that they might bind differently to the delta receptor than diprenorphine and buprenorphine.


Fig. 7. Chemical structure of agonist (−)-buprenorphine and structurally related antagonist (−)-diprenorphine.


