Mechanism of Down-regulation of Alpha-2 Adrenergic Receptor Subtypes

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Accepted for publication May 16, 1997

ABSTRACT

Long-term exposure to agonist down-regulates receptor expression for many G protein-coupled receptors. This decrease in receptor density could occur through either an increase in receptor degradation or a decrease in receptor synthesis. We studied the mechanism of down-regulation of the alpha-2A and alpha-2B adrenergic receptor subtypes transfected into the Chinese hamster ovary cell line as well as the alpha-2A receptor endogenous to the HT29 cell line. The rate constants for receptor appearance and disappearance were calculated from the recovery of receptor expression after irreversible inactivation of the existing receptor population with an alkylating agent. In the presence of the agonist norepinephrine, the receptor subtypes in all three cell lines down-regulated to about 50% with a half-time of 2.5 hr. When recovering in the presence of norepinephrine after irreversible inactivation, the rate of receptor degradation increased approximately 2-fold for all three cell lines with little change in the rate of synthesis. During this recovery, the transfected alpha-2A receptor exhibited a half-life of 3.0 hr, which agrees with the 2.7-hr half-time of down-regulation in the presence of norepinephrine. In contrast, the transfected alpha-2B receptor and the endogenous alpha-2A receptor had a half-life of 1.2 hr and 8.9 hr, respectively. For only the endogenous alpha-2A receptor, pertussis toxin increased the half-time of down-regulation to 9.8 hr, similar to the 8.9-hr receptor half-life in the presence of norepinephrine during recovery after irreversible inactivation. Our results indicate that the mechanism of down-regulation of the alpha-2A and -2B adrenergic receptor subtypes is an increase in the rate of receptor degradation.

The catecholamine neurotransmitters epinephrine and norepinephrine mediate their physiological responses through the family of adrenergic receptors. Three types or subfamilies of adrenergic receptors have been identified: the alpha-1, alpha-2 and beta. Within each of these subfamilies are receptor subtypes, including the subtypes of alpha-2 adrenergic receptors: alpha-2A, -2B and -2C (Bylund et al., 1994).

The expression of these receptors is not static and can change with disease, aging or therapeutic treatment. Alteration of receptor density can occur at any of the steps from gene transcription to degradation of the receptor protein itself. Continued agonist stimulation of a receptor population often causes a rapid reduction in response to the agonist, a phenomenon known as desensitization. Short-term desensitization is characterized as a rapid (minutes) and reversible uncoupling of the receptor-G protein complex mediated by receptor phosphorylation. This is followed by sequestration and internalization of receptors from the cell surface. Receptors are not lost during short-term desensitization because removal of agonist rapidly restores receptor function. Down-regulation, on the other hand, is defined as a decrease in receptor density and displays a much longer time course (hours) which is thought to result from an actual loss of receptors. Removal of agonist will allow recovery of receptor density, but this recovery takes longer, requiring synthesis of new receptors in most cases (Hein and Kobilka, 1995; Toews et al., 1991).

The mechanisms for down-regulation of G protein-coupled receptors are not well characterized but may involve the accelerated removal of receptor from the cell surface, a decrease in the rate of receptor synthesis or both. For example, studies of rat beta-1 and beta-2 adrenergic receptors in tissue culture suggest a long-term decrease in the rate of gene transcription and a subsequent decrease in mRNA levels (Hosoda et al., 1994, 1995). Down-regulation of rat beta receptors in vivo can occur through a net increase in disappearance (beta-1) or a concurrent increase in disappearance and decrease in appearance (beta-2) (Snively et al., 1985). Down-regulation of receptor density through a decrease in synthesis has been shown to occur with human muscarinic M3 receptors (Steel and Buckley, 1993) and rabbit alpha-1B adrenergic receptors (Izzo and Colucci, 1994).

Agonist-induced down-regulation of alpha-2 receptors has

ABBREVIATIONS: CHO, Chinese hamster ovary; EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; G protein, guanine nucleotide binding regulatory protein.
been demonstrated both in vivo and in vitro. In vivo treatment of rats with desipramine to block re-uptake of norepinephrine results in down-regulation of brain alpha-2 receptors to 50 to 60% of control, depending on the brain region studied (Barturen and Garcia-Sevilla, 1992). Treatment with clorgyline, a monoamine oxidase inhibitor, also results in down-regulation of alpha-2 receptors to 50% of control (Ribas et al., 1993). These studies found that down-regulation produces an increase in both receptor appearance and disappearance, with a greater increase in disappearance accounting for receptor down-regulation. Whereas these studies were successful in exploring the mechanism of alpha-2 receptor down-regulation in rats, the methods used to produce down-regulation were indirect, and no distinction among receptor subtypes was made. In vitro studies with the NG-108 cell line which endogenously expresses the rat alpha-2B receptor found that agonist treatment produces down-regulation to about 50% of control (Thomas and Hoffman, 1986). The human alpha-2A and -2B subtypes transfected into the CHO cell line exhibit only 20 to 25% down-regulation (to around 75% of control) with 100 μM epinephrine (Eason and Liggett, 1992). We have characterized agonist down-regulation of several alpha-2 receptor subtypes in vitro. The alpha-2C receptor endogenously expressed in the opossum kidney cell line down-regulates to about 50% of control with 0.3 μM norepinephrine (Pleus et al., 1993; Shreve et al., 1991). Likewise, the rat alpha-2B receptor stably transfected into the CHO cell line down-regulates to a similar extent with 0.3 μM norepinephrine (Cerutis et al., 1996). The human alpha-2A receptor endogenously expressed in HT29 cells or stably transfected into CHO cells also down-regulates to about 50% of control, but down-regulation of this subtype requires 30 μM norepinephrine, which is approximately 100-fold greater than that required to down-regulate the alpha-2B and -2C subtypes (Pleus et al., 1993; Shreve et al., 1991).

Little is currently known about the mechanism of agonist-induced down-regulation of alpha-2 receptor subtypes. Our previous studies showed that the human alpha-2A receptor undergoes agonist-induced down-regulation in a similar fashion whether endogenously expressed or stably transfected (Pleus et al., 1993). Since the rate of receptor synthesis for the transfected receptor is no longer under the control of the endogenous promoter, it seems unlikely that agonist-induced receptor down-regulation of the human alpha-2A receptor occurs through a decrease in the rate of synthesis of new receptors. Therefore, we hypothesized that receptor down-regulation occurs through an increase in the rate of receptor degradation. As reported here, we tested this hypothesis by comparing the rates of receptor appearance and disappearance in the absence and presence of norepinephrine by the method of receptor recovery from irreversible inactivation. Our results indicate that the mechanism of down-regulation of receptors is an increase in the rate of receptor degradation. In addition, the mechanism of initial down-regulation may be different from that of maintaining receptors in the down-regulated state, and this difference may be attributable, at least in part, to G protein coupling.

**Methods**

**Cell culture.** The HT29 human adenocarcinoma cell line was obtained from ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with high glucose, 10% fetal bovine serum (HyClone, Logan, UT) and 3 μg/ml Penn-G (Sigma Chemical Co., St. Louis, MO). CHO-K1 cells were transfected with either the human alpha-2A receptor (CHO-2A, kindly provided by Drs. Fraser and Venter, National Institutes of Health, Bethesda, MD (Jones et al., 1991; Fraser et al., 1989)) or the rat alpha-2B receptor (CHO-2B [Cerutis et al., 1996]) and maintained in Ham's F-12 medium (Gibco) supplemented with 10% fetal bovine serum (HyClone), 5 μg/ml Penn-G (Sigma) and 100 μg/ml genetin (Gibco). All cells were grown in a 5% CO₂ humid environment at 37°C.

**Drug treatment.** Cells were plated at a density of 100,000 cells/ml (CHO cells) or 200,000 cells/ml (HT29 cells), 4 ml total per dish, and treated on day 5 (CHO cells) or day 7 (HT29 cells) of culturing, when the monolayers approached confluence. Fresh medium was given on days 2 and 4 for CHO cells and on days 3 and 6 for HT29 cells. On the day of treatment, medium was aspirated and replaced with fresh medium containing either 10 μM irreversible alkylating compound EEDQ (Research Biochemicals International, Natick, MA) or ethanol vehicle control. After a 30-min incubation at 37°C, the cells were rinsed three times with 3 ml serum-free medium and then given fresh medium without or with norepinephrine (Research Biochemicals International) at a concentration of 30 μM for the alpha-2A receptor and 0.3 μM for the alpha-2B receptor. The cells were then placed back in the incubator and harvested at various times up to 72 hr depending on the receptor and cell line. A typical experiment consisted of control cells, cells incubated with norepinephrine alone, cells pretreated with EEDQ and allowed to recover in the absence of norepinephrine and cells pretreated with EEDQ and allowed to recover in the presence of norepinephrine. Cells treated with cycloheximide (ICN Biomedicals, Costa Mesa, CA) were plated and cultured as explained above. On the day of treatment, cells were treated with EEDQ as explained previously. Cells were then given fresh medium without or with cycloheximide (5 μg/ml), placed back in the incubator and harvested at various times up to 36 hr. For the saturation binding experiments, cells were treated with EEDQ and allowed to recover in the absence or presence of norepinephrine as explained previously and harvested at 32 hr for the CHO alpha-2A and 24 hr for the CHO alpha-2B receptors. For cells treated with pertussis toxin (List Biologicals, Camphill, CA), cells were first preincubated with 100 ng/ml pertussis toxin for 18 hr and incubated in the continued presence of pertussis toxin for the duration of the experiment.

**Radioligand binding assays.** Membranes were prepared for radioligand binding assays by rinsing and scraping a confluent monolayer of cells with ice-cold phosphate-buffered saline (137 mM NaCl/2.5 mM KC1/20 mM Na₃HPO₄) and pelleting cells by centrifugation at 1000 × g for 5 min at 4°C. Cell pellets were resuspended in ice-cold 50 mM Tris buffer, pH 8.0, and homogenized by a Tissumizer (Tekmar, Cincinnati, OH) at the maximum setting for 15 sec. A membrane pellet was obtained by centrifugation of the homogenate.

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>0 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
<th>36 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-2A</td>
<td>1.2 ± 1.2</td>
<td>5.0 ± 3.2</td>
<td>6.0 ± 2.5</td>
<td>5.2 ± 0.5</td>
<td>13.9 ± 1.7</td>
</tr>
<tr>
<td>CHO-2B</td>
<td>4.5 ± 1.1</td>
<td>6.0 ± 1.9</td>
<td>6.3 ± 2.0</td>
<td>8.6 ± 2.1</td>
<td>9.2 ± 2.9</td>
</tr>
</tbody>
</table>
TABLE 2  
Affinity of [3H]RX821002 for alpha-2A and -2B receptors after incubation of cells under various conditions  

For the saturation binding experiments, cells were treated with 10 μM EEDQ or ethanol vehicle (control). After removal of residual EEDQ, cells were allowed to recover in the absence or presence of norepinephrine (NE) (30 μM for alpha-2A and 0.3 μM for alpha-2B) and harvested at 32 hr for the CHO-2A and 24 hr for the CHO-2B cells. Comparison of affinities was done by a one-way analysis of variance on the geometric means.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Treatment</th>
<th>Affinity (Kd)</th>
<th>n</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-2A</td>
<td>− −</td>
<td>200 ± 10</td>
<td>4</td>
<td>.12</td>
</tr>
<tr>
<td></td>
<td>+ −</td>
<td>160 ± 15</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>189 ± 17</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>− +</td>
<td>169 ± 14</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CHO-2B</td>
<td>− −</td>
<td>1370 ± 50</td>
<td>3</td>
<td>.19</td>
</tr>
<tr>
<td></td>
<td>+ −</td>
<td>1380 ± 10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>1270 ± 50</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Time course and extent of norepinephrine down-regulation of alpha-2 adrenergic receptors. Confluent monolayers of HT29, CHO-2A and CHO-2B cells were incubated with norepinephrine (30 μM for alpha-2A receptors and 0.3 μM for alpha-2B receptors) and harvested at the indicated times. Each data point represents the mean of six or seven experiments ± S.E.M. The rate constants for appearance and disappearance calculated from these data are given in table 3.

Results  
The alkylating agent EEDQ has been shown to irreversibly block alpha-2 adrenergic receptors in vivo (Ribas et al., 1993; Barturen and Garcia-Sevilla, 1992; Hayashi et al., 1995; Durcan et al., 1994). To demonstrate the ability of this compound to irreversibly block alpha-2 receptors in tissue culture, CHO cells stably transfected with either the human alpha-2A (CHO-2A) or the rat alpha-2B (CHO-2B) receptor were treated with 10 μM EEDQ to inactivate the existing receptors. After rinsing to remove residual EEDQ, the cells were allowed to recover for various times in the presence of cycloheximide to block the synthesis of new receptors. Mem-
branes from the cells were then assayed for alpha-2 adrenergic receptor binding (table 1). In the presence of cycloheximide, very little recovery of specific binding occurred with either alpha-2A or -2B receptors during 36 hr, which indicated that EEDQ irreversibly inactivated these binding sites.

A single concentration of radioligand was used in most experiments to estimate changes in receptor expression. This method assumes that the affinity of the receptor for the ligand is not altered by the various drug treatments of the cells. To confirm the validity of this assumption, six-point saturation studies were done on cells receiving the various treatments. For both the CHO-2A and -2B receptors, cells treated with either norepinephrine, EEDQ or both norepinephrine and EEDQ displayed an alpha-2 receptor population with affinities for [3H]RX821002 not different than the alpha-2 receptor population in control cells (one-way analysis of variance, P > .10; table 2).

To assess the time course of alpha-2 receptor down-regulation, cell lines expressing the human alpha-2A and rat alpha-2B receptors were treated with norepinephrine and harvested at various time points up to 30 hr (fig. 1). We have previously shown that norepinephrine is about 100-fold more potent in down-regulating rat alpha-2B receptors than human alpha-2A receptors (Cerutis et al., 1996). The significance of this observation is not fully understood, but it may represent a fundamental difference in the way in which these receptors are regulated. Thus, to produce a comparable extent of down-regulation in the current studies, 30 μM norepinephrine was used to down-regulate alpha-2A receptors whereas only 0.3 μM norepinephrine was used to down-regulate alpha-2B receptors. We have previously found no significant down-regulation of the alpha-2A subtype after a 24-hr incubation with norepinephrine at a concentration of 0.3 μM (Cerutis et al., 1996; Shreve et al., 1991). For the alpha-2B subtype, increasing the concentration of norepinephrine from 0.3 μM to 30 μM does not increase the extent of down-regulation (Cerutis et al., 1996). Norepinephrine down-regulated the HT29 cells (HT29-2A) receptor to 57% of control levels after 24 hr. This decrease was consistent with a monoeponential decay model with a half-life of disappearance of 2.4 hr. The CHO-2A receptor exhibited a time course very similar to that of the same receptor expressed endogenously in HT29-2A. Norepinephrine caused this receptor to down-regulate to 54% of control levels after 24 hr with a half-life of disappearance of 2.7 hr. Norepinephrine similarly down-regulated the CHO-2B receptor to 46% of control levels after 24 hr with a half-life of 2.4 hr (table 3).

To determine whether down-regulation occurs through an increase in the rate of receptor degradation or a decrease in the rate of receptor synthesis (or both), the rate constants for receptor appearance (r) and disappearance (k) were deter-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Appearance (r)</th>
<th>Disappearance (k)</th>
<th>Receptor expression (r/k)</th>
<th>Half-life (hr)</th>
<th>r²</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>16.5 ± 4.1</td>
<td>0.291 ± 0.063</td>
<td>57</td>
<td>2.4</td>
<td>0.93</td>
<td>6</td>
</tr>
<tr>
<td>CHO-2A</td>
<td>14.0 ± 2.9</td>
<td>0.259 ± 0.045</td>
<td>54</td>
<td>2.7</td>
<td>0.98</td>
<td>6</td>
</tr>
<tr>
<td>CHO-2B</td>
<td>13.4 ± 4.7</td>
<td>0.289 ± 0.082</td>
<td>46</td>
<td>2.4</td>
<td>0.96</td>
<td>7</td>
</tr>
</tbody>
</table>

Fig. 2. Recovery of alpha-2 adrenergic receptors after irreversible blockade with EEDQ. Confluent monolayers of HT29, CHO-2A or CHO-2B cells were incubated with 10 μM EEDQ for 30 min and then rinsed three times with serum-free medium. Cells were then allowed to recover with fresh medium in the absence (○) or presence (●) of norepinephrine (NE) (30 μM for alpha-2A receptors and 0.3 μM for alpha-2B receptors) and harvested at various times through 24 or 72 hr. Each data point represents the mean of 4 to 13 determinations ± S.E.M. The rate constants for appearance and disappearance calculated from these data are given in table 4.
TABLE 4

Alpha-2 receptor expression and rate constants for appearance and disappearance after recovery from EEDQ treatment in the absence or presence of norepinephrine

Cells were treated with 10 μM EEDQ or ethanol vehicle (control). After removal of residual EEDQ, cells were allowed to recover in the absence or presence of norepinephrine (NE) (30 μM for alpha-2A and 0.3 μM for alpha-2B) and harvested at various times up to 72 hr. The data were fit to the repopulation equation to obtain values for \( r \) and \( k \). Goodness-of-fit was estimated from the correlation coefficient \( (r^2) \) and from the t-test which was not significant \( (P > .05) \) in all cases. Extent of receptor expression is determined by \( r/k \).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Appearance (r)</th>
<th>Disappearance (k)</th>
<th>Receptor expression (r/k)</th>
<th>Half-life</th>
<th>( r^2 )</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−NE</td>
<td>5.1 ± 0.6</td>
<td>0.054 ± 0.008</td>
<td>94</td>
<td>12.8</td>
<td>0.96</td>
<td>7</td>
</tr>
<tr>
<td>+NE</td>
<td>4.0 ± 0.4</td>
<td>0.078 ± 0.008*</td>
<td>51</td>
<td>8.9</td>
<td>0.99</td>
<td>9</td>
</tr>
<tr>
<td>CHO-2A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−NE</td>
<td>9.2 ± 1.1</td>
<td>0.106 ± 0.016</td>
<td>87</td>
<td>6.5</td>
<td>0.96</td>
<td>4</td>
</tr>
<tr>
<td>+NE</td>
<td>10.8 ± 1.8</td>
<td>0.232 ± 0.047*</td>
<td>47</td>
<td>3.0</td>
<td>0.92</td>
<td>4</td>
</tr>
<tr>
<td>CHO-2B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−NE</td>
<td>21.1 ± 1.8</td>
<td>0.209 ± 0.024</td>
<td>101</td>
<td>3.3</td>
<td>0.98</td>
<td>10</td>
</tr>
<tr>
<td>+NE</td>
<td>24.4 ± 7.6</td>
<td>0.601 ± 0.215*</td>
<td>40</td>
<td>1.2</td>
<td>0.89</td>
<td>13</td>
</tr>
</tbody>
</table>

For recovery of the HT29–2A receptor population in cells without norepinephrine, the rate constant for appearance was 5.1%/hr and the rate constant for disappearance was 0.054 hr\(^{-1}\) (table 4). For recovery of receptors in norepinephrine-treated cells, the rate constant for appearance was 4.0%/hr and for disappearance 0.078 hr\(^{-1}\). The change in the rate constant for appearance caused by norepinephrine was not significant, whereas the change in the rate constant for disappearance was statistically significant (Student’s t-test, \( P < .05 \)). HT29-2A receptors, in the absence of agonist, exhibited a disappearance half-life (ln2/\( k \)) of 12.8 hr, whereas the receptors in the presence of agonist had a shorter half-life of 8.9 hr (table 4). The extent of down-regulation can be calculated from the values \( r \) and \( k \) (\( R_{\text{down}} = r/k \), see “Discussion”). The \( r/k \) value was 51% of control and agrees well with the extent of down-regulation seen in figure 1 (57%, table 3). Furthermore, this down-regulated state appears to occur through an increase in the rate of disappearance, whereas the rate of appearance remains constant.

CHO-2A receptors recovered to an extent similar to the HT29-2A receptors. EEDQ treatment produced a blockade >95%, and recovery of receptor binding in the presence of agonist approached 50% of control levels (fig. 2). However, the rate constants for appearance and disappearance in CHO-2A cells were very different from those in the HT29 cells, as indicated by the different time scales in the upper and middle panels of figure 2. For cells allowed to recover in the absence of norepinephrine, the rate constant for alpha-2A receptor appearance was 9.2%/hr and that for disappearance was 0.11 hr\(^{-1}\). For recovery in the presence of norepinephrine, the rate constant for appearance was 11%/hr and for disappearance was 0.23 hr\(^{-1}\), giving an extent of down-regulation (\( r/k \)) of 47% of control (table 4). Consistent with our findings for the HT29–2A receptors, the change in the rate constant for appearance caused by norepinephrine was not significant, whereas the change in the disappearance rate constant was significant \( (P < .05) \). The rate of turnover for the alpha-2A receptor in CHO cells was more rapid with receptor half-lives of 6.5 hr and 3.0 hr in the absence and presence of norepinephrine, respectively, compared with 12.8 hr and 8.9 hr for HT29 cells (table 4). When comparing the rate constants for both HT29 and CHO-2A cells, the rates of both appearance and disappearance in the HT29 cells were significantly slower than in the CHO-2A cells \( (P < .05) \) for all comparisons). Despite this difference, down-regulation occurs predominately through an increase in receptor disappearance for both cell lines.

Similar to the alpha-2A receptor, EEDQ treatment inactivated >95% of alpha-2B receptors in the CHO-2B cells. In agreement with figure 1, recovery of receptors in the presence of agonist produced a new steady-state level around 40% of the control population (fig. 2). The rate constant for appearance in cells without norepinephrine was 21%/hr and in norepinephrine-treated cells was 24%/hr. Receptor disappearance in untreated cells occurred at a rate of 0.21 hr\(^{-1}\), corresponding to a receptor half-life of 3.3 hr. For norepinephrine-treated cells, the disappearance rate constant was 0.60 hr\(^{-1}\), corresponding to a receptor half-life of 1.2 hr. Similar to the alpha-2A receptor, the alpha-2B receptor had a statistically significant increase in the rate of disappearance in the presence of norepinephrine \( (P < .05) \) with no significant change in the rate of appearance (table 4). Although down-regulation of the alpha-2B receptor and alpha-2A receptor occurs through an increase in the rate of receptor disappearance, the alpha-2B receptor has a shorter half-life and thus a more rapid turnover rate.

We next studied the effects of G protein coupling on receptor down-regulation. It has been shown for the beta-2 adrenergic receptor that G protein coupling (but not effector activation) is necessary for down-regulation (Lohse, 1993). In contrast, blocking G protein coupling to alpha-2 receptors with pertussis toxin had no effect on the extent of down-regulation in NG-108 cells which endogenously express the rat alpha-2B receptor (Thomas and Hoffman, 1986), al-
though time courses were not compared for down-regulation in either the absence or presence of pertussis toxin. Pertussis toxin ADP-ribosylates G Moff proteins, thereby preventing their interaction with the receptor. To determine the effect of G protein coupling on the time course of down-regulation, HT29 cells were treated with pertussis toxin 18 hr before, as well as during, norepinephrine-induced down-regulation (fig. 3). Down-regulation in the presence of pertussis toxin occurred with a half-life of disappearance of 9.8 hr (table 5), which was significantly longer than the 2.1 hr half-time of disappearance in the absence of pertussis toxin (P < .05). This result suggests that receptor down-regulation in the presence of norepinephrine may be regulated by G protein coupling in the HT29 cell line. Norepinephrine-induced down-regulation of CHO-2A receptors in the presence of pertussis toxin occurred with a rate of appearance of 11%/hr and a rate of disappearance of 0.20 hr⁻¹ (fig. 3), which do not differ significantly from the rate constants observed for either down-regulation or recovery in the presence of norepinephrine. Pertussis toxin also failed to alter the time course of down-regulation of CHO-2B receptors. In the presence of pertussis toxin, down-regulation occurred with an appearance rate constant of 11%/hr and a disappearance rate constant of 0.27 hr⁻¹, which did not differ statistically from those obtained in the absence of pertussis toxin. Pertussis toxin treatment alone, however, decreased receptor expression by 61% ± 2% of control in CHO-2B cells, which indicated a role for G proteins in the regulation of receptor expression. In contrast, pertussis toxin treatment had little effect on receptor expression in HT29 cells (96% ± 10%) and slightly increased expression in CHO-2A cells (116% ± 8%) (n = 2 or 3).

To confirm that a decrease in the second messenger cyclic AMP is not involved in the effects of incubation with norepinephrine, cells were incubated in the presence of 25 μM dibutyryl-cyclic AMP (table 6). After a 24 hr incubation, dibutyryl-cyclic AMP alone did not affect receptor expression nor was it able to alter norepinephrine-induced down-regulation of receptor expression. These results indicate that cyclic AMP does not have a significant role in receptor down-regulation.

Discussion

It is well established that the expression of many G protein-coupled receptors is consistent with a model in which the rate of receptor appearance is a zero-order process (rate is constant) and the rate of disappearance is a first-order process (rate is proportional to the density of existing receptors) (Neve and Molinoff, 1986). With these assumptions, the change in the rate constants for appearance and/or disappearance when going from one steady state level of receptor expression to a new steady state level can be modeled by the equation: 

\[ R_t = R_0 e^{-kt} + R_a e^{-kt} \]

where \( R_t \) is the density of receptors at time \( t \), \( R_0 \) is the density of receptors at time 0, \( k \) and \( r \) are the rate constants for appearance and disappearance, respectively, and \( R_a \) is the density of receptors at time 0 (Neve and Molinoff, 1986). As time approaches infinity, the term \( e^{-kt} \) approximates zero, and thus the receptor level at steady state, \( R_m \), approaches \( r/k \). Thus, to maintain a new steady-state level, a net change in receptor appearance or disappearance must occur (Snively et al., 1985). This model is based on the assumption that \( r \) and \( k \) do not change during the transition from one steady state level to another. When an irreversible inhibitor is used, this model also assumes that the blockade of existing receptors is permanent (Neve and Molinoff, 1986).

The time course and extent of down-regulation in HT29 and CHO-2A cells were very similar, with a half-life of receptor disappearance of about 2.5 hr. The loss of about 50% of the receptors in both cases agrees well with our previous results (Cerutis et al., 1996). By contrast, another study with CHO-2A cells reported agonist-induced down-regulation to only 75% of control using 100 μM epinephrine (Eason and Liggett, 1992). Norepinephrine at a concentration of 0.3 μM was sufficient to down-regulate the rat alpha-2B receptor stably transfected into the CHO cell line to 46% of control. This is in agreement with our previous results (Cerutis et al., 1996), but again in contrast to the results of Eason and
Liggett (1992), where CHO cells transfected with the human alpha-2B receptor down-regulated to only 80% of control with 100 μM epinephrine. The relatively small extent of down-regulation obtained by Eason et al. (1992) could reflect the different agonists (epinephrine vs. norepinephrine) or the choice of concentration used to induce down-regulation (100 μM vs. 30 μM or 0.3 μM). In preliminary studies we found a biphasic response of the CHO transfected opossum alpha-2C receptor to norepinephrine, such that increasing concentrations of norepinephrine greater than 0.3 μM result in a decrease in the extent of down-regulation or even an up-regulation of receptors (data not shown).

HT29 cells had a time of recovery after EEDQ blockade that was significantly slower than the CHO-2A cells (approximately 50 hr compared with 15 hr) as reflected in the lower rate constant for disappearance in the HT29 cell line. The rate constant for appearance was significantly lower in HT29 cells as well. This can be explained partially by the difference in cell types, because the time for cell division in the HT29 cells is approximately 2-fold greater than in the CHO cell line (Forgue-Lafitte et al., 1989; Kao and Puck, 1975). A difference in receptor stability has also been observed for rat brain alpha-2 receptors in which the receptor half-life varied from 1 to 4 days depending on the brain region (Barturen and Garcia-Sevilla, 1992). Despite differences in the absolute values of the rate constants, both HT29-2A and CHO-2A receptors exhibit an increase in the rate constant for disappearance during norepinephrine-induced down-regulation.

Studying the same receptor (human alpha-2A) expressed endogenously (HT29-2A) or transfected (CHO-2A), as well as two different subtypes expressed in the same cell line (CHO-2A vs. CHO-2B), may help determine whether the mechanism for down-regulation occurs at the level of protein expression or depends on the cellular background in which the receptor is expressed, including the cell-specific regulation of gene transcription. Down-regulation of alpha-2A and -2B receptors in the presence of norepinephrine occurs with a similar time course irrespective of receptor subtype or cell line. Based on receptor recovery in the absence or presence of agonist after irreversible inactivation, this down-regulation occurs largely through an increase in the rate of receptor disappearance. In previous studies, we have been unable to find evidence for a decrease in mRNA levels after agonist pretreatment in either CHO-2A or CHO-2B cells (Bylund et al., 1995), which supports an increase in receptor degradation rather than a decrease in receptor synthesis as the mechanism for down-regulation.

In a system going from one steady state level of expression to a new steady state, the repopulation equation is valid for the determination of the rate constants for the new steady state. As such, the down-regulation time courses presented in figure 1 can be fit to the repopulation equation to obtain rate constants for receptor appearance and disappearance in the presence of norepinephrine (table 3). Of particular interest is the observation that the receptors in all cases down-regulate with very similar rate constants for appearance and disappearance. This is in contrast to the rates of appearance and disappearance based on the data from receptor recovery in the presence of norepinephrine after EEDQ blockade (table 4). The observation that all three cell lines have similar rate constants calculated from down-regulation time courses (table 3) but different rate constants calculated from recovery time courses (table 4) cannot be easily explained. One possibility is the accessibility of existing, agonist-occupied cell-surface receptors to the G protein pool as compared with the newly synthesized, agonist-occupied receptors appearing on the cell surface. Figure 4 compares the observed time courses of down-regulation in the presence of norepinephrine and pertussis toxin, which blocks G protein coupling, with predicted curves of down-regulation based on the data for receptor recovery from EEDQ treatment in the presence of norepinephrine alone. For the HT29-2A and CHO-2A receptors, the actual time course of down-regulation in the presence of pertussis toxin is similar to the predicted time course. In contrast, the CHO-2B receptors have a predicted time course of down-regulation in the presence of pertussis toxin much quicker than the actual time course, as evidenced by the
increased slope of the predicted line compared with the observed line. Interestingly, using pertussis toxin to block G protein coupling during down-regulation in the presence of norepinephrine increased rate constants similar to those during recovery after EEDQ treatment for the HT29-2A receptors. This is reflected in the 9.8-hr receptor half-life in the presence of norepinephrine alone (table 4), and in contrast to the 2.4-hr half-life in the absence of pertussis toxin (table 5). This suggests that down-regulation of HT29–2A receptors is modulated by G protein coupling, whereas recovery after irreversible inactivation is not.

Pertussis toxin treatment had no effect on receptor expression in HT29 cells and caused a slight increase in receptor expression for CHO-2A cells. In contrast, pertussis toxin treatment alone down-regulated the density of alpha-2B receptors by about 40% while not altering the rate constants for norepinephrine-induced down-regulation (table 5). The decrease in CHO-2B receptor expression with pertussis toxin alone is in disagreement with two previous studies which have reported an increase in alpha-2B receptor expression upon pertussis toxin treatment. Thomas and Hoffman (1986) reported that pertussis toxin increases the expression of rat alpha-2B receptors endogenously expressed in NG-108 cells by about 50% while not affecting the extent of down-regulation. Shi and Deth (1994) reported that pertussis toxin treatment increases the density of rat alpha-2B receptors transfected into PC-12 cells by 30%. They suggested that pertussis toxin treatment increases the affinity of the antagonist rauwolscine (the radioligand used in their experiments) to receptors thereby increasing the extent of maximal binding. To test whether the choice of radioligand determines the effects of pertussis toxin on receptor expression, we used [3H]rauwolscine as the radioligand and found no differences in either the affinity or density of receptors after pertussis toxin treatment when compared with receptor binding with [3H]RX821002 as the radioligand (data not shown).

The rate constants for appearance and disappearance obtained from the down-regulation time course differ from those obtained from the recovery data, which suggests that the mechanism for receptor down-regulation is not necessarily the same mechanism for maintaining receptors in the down-regulated state. This difference may be attributable, in part, to G protein coupling, because inhibition of G protein coupling with pertussis toxin treatment in the HT29 cell line produces rate constants for agonist down-regulation similar to those for receptor recovery in the presence of agonist. In addition, recovery of receptors after EEDQ blockade is not affected by the presence of pertussis toxin in any of the three cell lines (data not shown). When comparing the two transfected cell lines (CHO-2A and CHO-2B), pertussis toxin had no effect on the rate constants for down-regulation. Taken together, these data suggest not less than three distinct rates for receptor disappearance. The first is the unoccupied receptor (disappearance rate constant from recovery in the absence of agonist after EEDQ treatment). The second is the agonist-bound receptor not coupled to G protein (disappearance rate constant from recovery in the presence of agonist after EEDQ treatment, or during down-regulation in the presence of agonist and pertussis toxin). The third is that of the agonist-bound receptor in association with G proteins (disappearance rate constant from down-regulation in the presence of agonist).

References


Down-regulation of Alpha-2 Receptors


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