Adrenergic agonists induce heterologous sensitization of adenylate cyclase in NS20Y-D_{2L} cells

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Abstract Adenylate cyclase activity in NS20Y cells expressing D_{2L} dopamine receptors was examined following chronic treatment with norepinephrine and epinephrine. Initial acute experiments revealed that both norepinephrine and epinephrine inhibited forskolin-stimulated cyclic AMP accumulation via D_2 receptors. Furthermore, chronic (18 h) activation of D_2 dopamine receptors by norepinephrine or epinephrine induced a marked increase (>10-fold) in subsequent forskolin-stimulated cyclic AMP accumulation. This heterologous sensitization of adenylate cyclase activity was blocked by D_2 dopamine receptor antagonists and by pertussis toxin pretreatment. In contrast, concurrent activation of $G\alpha_s$ or adenylate cyclase did not appear to alter noradrenergic agonist-induced sensitization. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: D_2 dopamine receptor; Heterologous sensitization; Norepinephrine; Epinephrine; NS20Y neuroblastoma; Supersensitization

1. Introduction

Dopaminergic systems have been implicated in the pathology and pharmacotherapy of many central nervous system disorders. Furthermore, evidence suggests that the noradrenergic system may also modulate dopamine signaling. For example, noradrenergic neurons project to several brain regions that contain both norepinephrine uptake sites and D_2-like dopamine receptors, including the ventral tegmental area, amygdala, and cortex [1–4]. Furthermore, in vitro studies have provided direct evidence that norepinephrine is an agonist at D_2-like dopamine receptors [5–8]. Norepinephrine stimulates D_2 dopamine receptor-mediated inhibition of cyclic AMP accumulation and high affinity GTPase activity in rat striatal membranes [5,8]. Studies examining recombinant dopamine receptors in heterologous expression systems report that norepinephrine has agonist activity at both D_2 and D_4 dopamine receptors [6,8].

The acute activation of D_2-like dopamine receptors is linked to inhibition of adenylate cyclase (EC 4.6.1.1) activity, however, several studies have shown that both sub-acute (2 h) and chronic (18 h) activation of D_2 and D_4 receptors induces heterologous sensitization (supersensitization) of adenylate cyclase activity [9–15]. Heterologous sensitization is characterized by enhanced drug-stimulated cyclic AMP accumulation in cells that were previously exposed to D_2 dopamine receptor agonists. Short-term (2 h) D_2 dopamine receptor-mediated sensitization of adenylate cyclase is prevented by coincubation with D_2 dopamine receptor antagonists and by pertussis toxin treatment [13,16], however, the exact molecular mechanisms of heterologous sensitization remain unknown.

Because D_2 dopamine receptor-induced heterologous sensitization leads to an upregulation of the cyclic AMP system, such drug-induced neuroadaptations may be associated with neuropsychiatric disorders that may involve dopamine and norepinephrine signaling [17–19]. The present study was designed to examine the ability of norepinephrine and epinephrine to induce heterologous sensitization in NS20Y neuroblastoma cells expressing the long (444-amino acid) form of D_2 dopamine receptor (D_{2L}). We now report that chronic treatment of NS20Y neuroblastoma cells expressing the D_{2L} dopamine receptor (NS20Y-D_{2L}) with norepinephrine or epinephrine markedly enhanced subsequent forskolin-stimulated cyclic AMP accumulation. This long-term heterologous sensitization did not occur in wild-type NS20Y cells and was blocked by D_2 dopamine receptor antagonists or by pertussis toxin pretreatment. Furthermore, the effects of persistent agonist activation of D_2 dopamine receptors were not altered by co-activation of the endogenous $G\alpha_s$-coupled β adrenergic receptor or by direct activation of adenylate cyclase.

2. Materials and methods

2.1. Materials

[^{3}H]Cyclic AMP (25 Ci/mmol) was purchased from NEN Life Science Products (Boston, MA, USA) and [^{3}H]spiperone (104 Ci/mmol) from Amersham Life Sciences (Arlington Heights, IL, USA). Quinpirole, epinephrine, norepinephrine, (+)-butaclamol, propranolol, spiperone, and forskolin were purchased from Research Biochemicals International (Natick, MA, USA). Dopamine (3-hydroxytyramine), isoproterenol, pertussis toxin, growth media and most other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Production and maintenance of cell lines

Transfection of NS20Y cells with the D_{2L} vector was carried out by calcium phosphate precipitation as described previously [13]. Briefly, pcDNA1-D_{2L} (20 μg) and pBabe Puro (2 μg) were mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2×BBS (50 mM *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM $NaHPO_4$) was added. The mixture was incubated for 25 min and added dropwise to exponentially growing NS20Y cells in a 10-cm tissue culture

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Abbreviations: D_{2L}, long (444-amino acid) form of D_2 dopamine receptor; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NS20Y-D_{2L} cells, NS20Y neuroblastoma cells expressing the D_{2L} dopamine receptor

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plate. Transfectants were isolated and screened by [^{3}H]spiperone binding. NS20Y-D_{2L} cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% calf bovine serum (CBS), penicillin/streptomycin, and puromycin (2 μg/ml). Cells were grown in a humidified incubator at 37°C in the presence of 10% CO_2.

2.3. Cyclic AMP accumulation assays

For sensitization assays, confluent cells in 48-well tissue culture clusters were preincubated for 18 h in DMEM assay buffer (DMEM supplemented with 5% FBS and 5% CBS in the presence of drugs at 37°C in a humidified incubator with 10% CO_2). For pertussis toxin treatment experiments, cells were initially treated with pertussis toxin (300 ng/well) in a volume of 200 μl for 2 h. After this 2-h incubation, 200 μl of a 20-μM agonist solution (final agonist concentration, 10 μM) was added to the wells which already contained pertussis toxin (the final concentration of pertussis toxin was 150 ng/ml in a total of 400 μl) and the cells were incubated for an additional 18 h. Following all 18-h drug treatments, the cells were washed three times for 3–4 min each with 200 μl EBSS assay buffer (Earle's balanced salt solution, containing 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 2% CBS and 0.02% ascorbic acid). To quantify sensitization of adenylate cyclase, forskolin (10 μM) was added in the presence of 1 μM spiperone to preclude acute effects of D_2-like dopamine receptor activation by residual agonist [13]. For acute experiments, cells were preincubated with 200 μl EBSS assay buffer for 10 min and placed on ice. Agonists in the absence or presence of antagonists were added to wells prior to the addition of forskolin (10 μM). Incubations were carried out for 15 min at 37°C and the assay buffer was decanted. The culture plates were placed on ice and lysed with 3% trichloroacetic acid (100 μl). The 48-well plates were stored at 4°C for at least 1 h before quantification of cyclic AMP.

2.4. Quantification of cyclic AMP

Cyclic AMP was quantified using a competitive binding assay [20], with minor modifications [16]. Duplicate samples of the 3% trichloroacetic acid cell lysates (2–10 μl) were added to reaction tubes each containing cyclic AMP assay buffer (100 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA). [^{3}H]Cyclic AMP (1 nM final concentration) was added to each assay, followed by cyclic AMP-binding protein (~100 μg of crude adrenal extract in 500 μl of cyclic AMP buffer). The reaction tubes were incubated on ice for 2 h, and the samples were then harvested by filtration (Whatman GF/C filters) using a 96-well Packard Filtermate cell harvester. Filters were allowed to dry, and 40 μl of Micoscint 0 scintillation fluid was added to each sample well. Radioactivity on the filters was determined using a Packard TopCount scintillation counter. Cyclic AMP concentrations in each sample were estimated in duplicate from a standard curve ranging from 0.1 to 100 pmol cyclic AMP/assay. Dose–response curves for cyclic AMP accumulation were analyzed by non-linear regression using the program GraphPad Prism (San Diego, CA, USA). All values for cyclic AMP accumulation are expressed as pmol cyclic AMP per well.

3. Results

3.1. Acute regulation of cyclic AMP accumulation in NS20Y-D_{2L} cells

We examined the ability of selected drugs to acutely modulate cyclic AMP accumulation in NS20Y-D_{2L} cells. The D_2 dopamine receptor agonist, quinpirole, inhibited forskolin-stimulated cyclic AMP accumulation, and the inhibition was blocked completely by the D_2 dopamine antagonist, spiperone (Fig. 1a). In contrast, propranolol did not alter the maximal quinpirole response in NS20Y-D_{2L} cells (Fig. 1a). Similarly, the natural ligand dopamine (10 μM) inhibited forskolin-stimulated cyclic AMP accumulation by 94 ± 2% ($n = 3$) and 93 ± 2% ($n = 3$) in the absence and presence of propranolol, respectively. We then examined the ability of epinephrine and norepinephrine to modulate cyclic AMP accumulation in

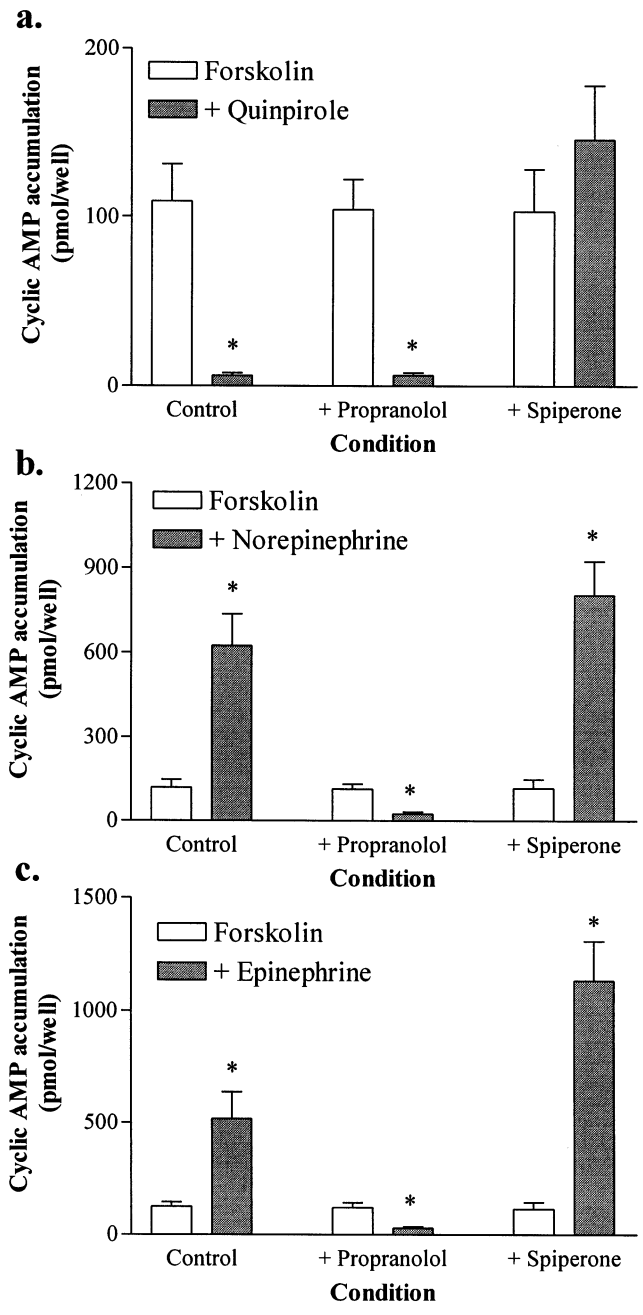

Fig. 1. Acute modulation of forskolin-stimulated cyclic AMP accumulation in NS20Y-D_{2L} cells. Cyclic AMP accumulation in NS20Y-D_{2L} cells was stimulated with 10 μM forskolin in the absence or presence of the indicated agonist (10 μM). a = quinpirole, b = norepinephrine, and c = epinephrine. Where indicated, some experiments were completed in the presence of 1 μM propranolol or 1 μM spiperone. Cyclic AMP accumulation assays were carried out for 15 min at 37°C and reactions were terminated as described in Section 2. Data shown are the mean ± standard error of five to eight independent experiments, each assayed in duplicate. *$P < 0.05$ compared to vehicle exposed cells (Student's t-test).

NS20Y-D_{2L} cells. In the absence of forskolin, the addition of norepinephrine and epinephrine produced no significant effect on cyclic AMP accumulation (data not shown). In contrast, when norepinephrine or epinephrine was added in the presence of forskolin, each drug produced a significant increase in cyclic AMP accumulation when compared to forsko-

lin alone (Fig. 1b,c). The addition of the β adrenergic antagonist propranolol prevented this increase and revealed that both norepinephrine and epinephrine had significant intrinsic activity for inhibiting forskolin-stimulated cyclic AMP accumulation. Specifically, in the presence of propranolol, norepinephrine and epinephrine inhibited forskolin-stimulated cyclic AMP accumulation by 76 ± 7% ($n = 5$) and 77 ± 6% ($n = 6$), respectively (Fig. 1b,c). In experiments that examined the effects of the D_2 dopamine antagonist spiperone on drug-modulated cyclic AMP accumulation, spiperone tended to potentiate both norepinephrine- and epinephrine-stimulated cyclic AMP accumulation (Fig. 1b,c).

3.2. Sensitization of forskolin-stimulated cyclic AMP accumulation in NS20Y-D_{2L} cells

Long-term (18 h) pretreatment of NS20Y-D_{2L} cells with 10 μM norepinephrine, 10 μM epinephrine, or 10 μM quinpirole resulted in marked sensitization of forskolin-stimulated cyclic AMP accumulation (Fig. 2a). In contrast, pretreatment with dopamine (10 μM) alone did not produce sensitization, presumably due to degradation under the culture conditions used (see [12]). The EC_{50} values for heterologous sensitization by norepinephrine and epinephrine were 2.3 ± 0.3 μM ($n = 3$) and 2.1 ± 0.7 μM ($n = 3$), respectively. An experiment with the synthetic D_2 agonist quinpirole revealed an EC_{50} value of 0.02 μM ($n = 1$) which is similar to a value reported in our previous study of short-term heterologous sensitization in NS20Y-D_{2L} cells [16]. Similar treatments of wild-type (untransfected) NS20Y neuroblastoma cells did not alter subsequent forskolin-stimulated cyclic AMP accumulation. Specifically, cyclic AMP accumulation values expressed as a percentage of vehicle-treated cell values were 100 ± 2% ($n = 7$), 105 ± 11% ($n = 7$), and 108 ± 7% ($n = 7$) in quinpirole-, epinephrine-, and norepinephrine-treated cells, respectively. We then examined D_2 dopamine receptor specificity and found that the effects of quinpirole, norepinephrine, or epinephrine treatment on subsequent forskolin-stimulated cyclic AMP accumulation in NS20Y-D_{2L} cells were blocked by the D_2 dopamine receptor antagonist, spiperone (Fig. 2b–d).

We next investigated the role of G protein-regulated pathways in drug-induced sensitization of adenylate cyclase. Pretreatment with pertussis toxin completely blocked long-term heterologous sensitization of forskolin-stimulated cyclic AMP accumulation mediated by quinpirole, and also prevented nor-

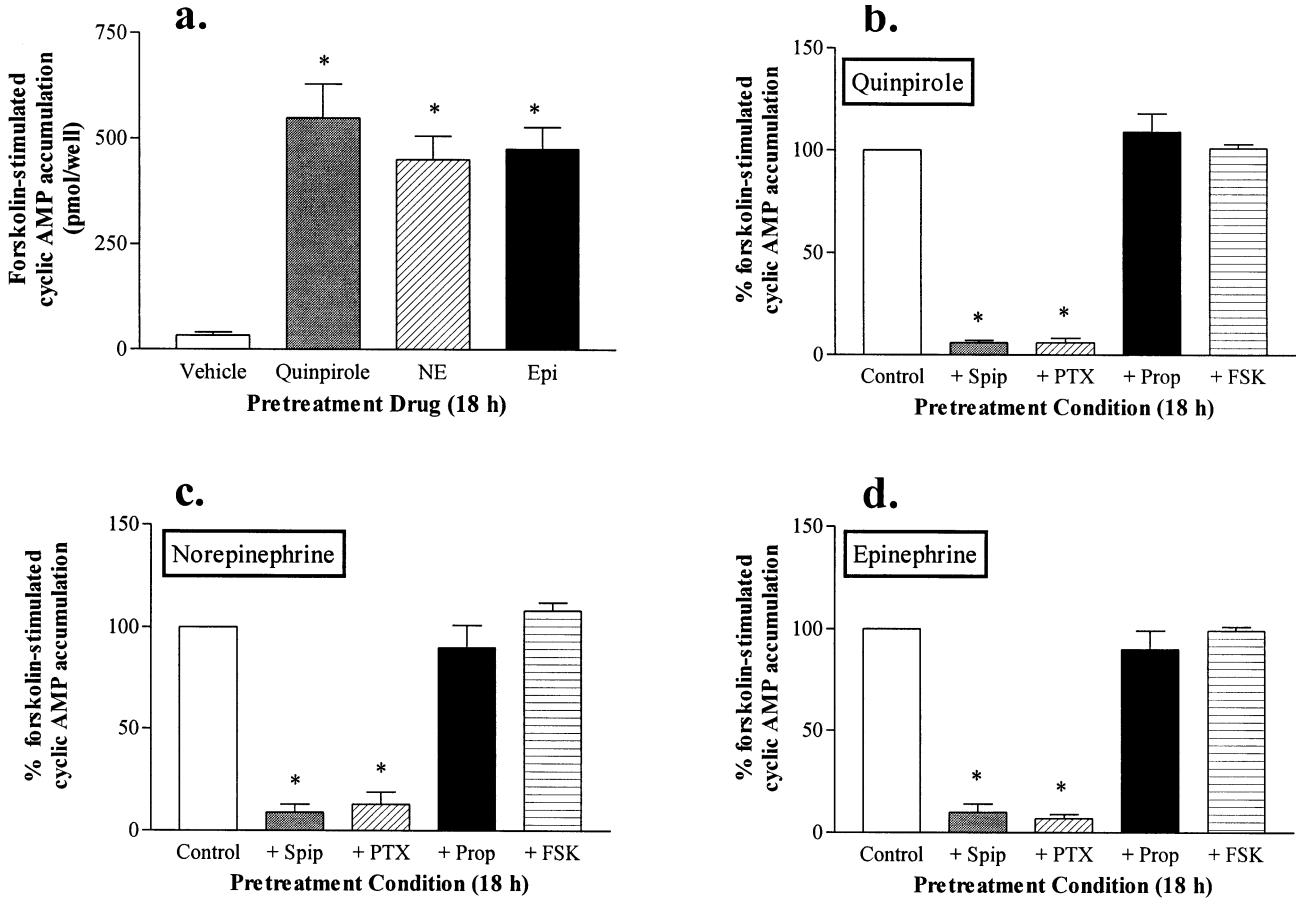

Fig. 2. Long-term agonist-induced heterologous sensitization of forskolin-stimulated cyclic AMP accumulation. a: NS20Y-D_{2L} cells were treated with vehicle or the indicated agonist (10 μM) for 18 h at 37°C and the response to forskolin (10 μM for 15 min) was then measured. b–d: Agonist (10 μM) pretreatments (18 h) were completed in the absence (Control) or presence of 1 μM spiperone (+Spip), 1 μM propranolol (+Prop), 10 μM forskolin (+FSK), or following treatment with pertussis toxin (+PTX). For pertussis toxin treatment, cells were initially treated with pertussis toxin (300 ng/well) for 2 h, followed by an 18-h incubation with agonist in the presence of pertussis toxin (150 ng/ml). Following all drug treatments, cells were washed extensively with EBSS, and cyclic AMP accumulation was stimulated with forskolin (10 μM) for 15 min. Data in b–d are expressed as the percentage of forskolin-stimulated cyclic AMP accumulation in cells treated with agonist alone (control). Data shown are the mean ± standard error of three to nine independent experiments, each assayed in duplicate. *$P < 0.001$ compared to vehicle-treated (a) or control (b–d) cells (Dunnett's post hoc ANOVA).

epinephrine- and epinephrine-induced sensitization of forskolin-stimulated cyclic AMP accumulation (Fig. 2b–d). We then compared drug-induced sensitization in the absence and presence of the β adrenergic receptor antagonist, propranolol, to examine the potential role of $G\alpha_s$-stimulated cyclic AMP accumulation on $G\alpha_{i/o}$-induced heterologous sensitization. The results of our studies demonstrate that antagonism of β adrenergic receptor activation did not alter heterologous sensitization induced by norepinephrine and epinephrine (Fig. 2c,d). Similarly, propranolol did not alter quinpirole-mediated sensitization (Fig. 2b). Additional studies revealed that direct activation of adenylate cyclase by 10 μM forskolin during agonist pretreatment does not alter heterologous sensitization (Fig. 2b–d).

4. Discussion

The experiments described above demonstrate that norepinephrine and epinephrine are agonists at recombinant D_2 dopamine receptors. Our results are in agreement with studies describing the ability of norepinephrine to activate D_2 dopamine receptors for inhibition of cyclic AMP accumulation [5] and to stimulate GTPase activity [8] in rat striatal membranes. These inhibitory effects of norepinephrine or epinephrine were readily observed in the presence of propranolol, indicating that the acute action of each drug on β adrenergic receptors is quite robust in the presence of forskolin. This observation is consistent with the synergistic action of forskolin and $G\alpha_s$ on adenylate cyclase activity following β adrenergic receptor activation [21].

In the present study we observed that long-term activation of D_2 dopamine receptors by quinpirole, norepinephrine, or epinephrine resulted in heterologous sensitization of forskolin-stimulated cyclic AMP accumulation. This heterologous sensitization was prevented when agonist pretreatments were combined with the D_2 antagonist spiperone. In agreement with short-term heterologous sensitization studies examining D_2-like receptors [12,13,16], we found that pretreatment with pertussis toxin completely blocked long-term heterologous sensitization of forskolin-stimulated cyclic AMP accumulation. Our results are also consistent with the report that pertussis toxin treatment blocks long-term sensitization of membrane adenylate cyclase activity by D_2 dopamine receptor agonists in Ltk-D_{2S} cells [9]. In addition, the present results implicate the activation of $G\alpha_{i/o}$ proteins (via D_2 receptors) in sensitization following chronic treatment with the adrenergic agonists, norepinephrine and epinephrine.

We also examined the effects of concurrent stimulation of adenylate cyclase during long-term heterologous sensitization. Our studies revealed that norepinephrine- and epinephrine-induced heterologous sensitization was not altered in the presence of propranolol (see Fig. 2c,d) suggesting that activation of the β adrenergic receptor-$G\alpha_s$-adenylate cyclase signaling pathway does not alter long-term heterologous sensitization. Furthermore, it was also demonstrated that heterologous sensitization is not affected by direct activation of adenylate cyclase using forskolin (Fig. 2b–d). Together, these observations suggest that activation of the protein kinase A pathway does not alter D_2 dopamine receptor-induced supersensitization of adenylate cyclase in NS20Y-D_{2L} cells. This result contrasts with an earlier study in Ltk-D_{2S} cell membranes demonstrating that activators of protein kinase A may enhance long-term D_2 agonist-mediated sensitization [9]. We have previously shown, however, that direct activation of protein kinase A or activation of the adenylate cyclase pathway *via* $G\alpha_s$-coupled receptors does not alter short-term D_2 dopamine receptor-mediated sensitization of adenylate cyclase activity in intact cells [13]. More recently, we have reported that direct activation of adenylate cyclase with forskolin does not alter short- or long-term heterologous sensitization by D_4 dopamine receptors in human embryonic kidney (HEK293) cells [12].

We chose NS20Y cells for the current studies of D_2 dopamine receptor function because they are of neuronal origin and have many features characteristic of striatal cholinergic cells [22]. We have previously characterized the D_2 dopamine receptor pharmacology in NS20Y-D_{2L} cells [16], and in the present study we examined the ability of norepinephrine and epinephrine to modulate D_2 dopamine receptor activity. Norepinephrine and epinephrine were agonists in experiments examining the effects of both acute and chronic activation of D_2 dopamine receptors in NS20Y-D_{2L} cells. Moreover, chronic activation of D_2 dopamine receptors by norepinephrine or epinephrine resulted in a marked degree of heterologous sensitization of adenylate cyclase that was similar to the heterologous sensitization produced by the D_2-selective agonist, quinpirole (Fig. 2a). Further, norepinephrine and epinephrine are also agonists at the dopamine D_4 receptor [6,7], which is expressed in brain areas that receive noradrenergic innervation [23]. Together, these observations suggest that crosstalk among noradrenergic neurons and neurons expressing D_2-like dopamine receptors may be important for interpretation of in vivo biochemical and behavioral studies.

The observation that norepinephrine is an agonist at D_2-like receptors may be important in psychiatric disorders that involve dopamine and norepinephrine function such as schizophrenia, depression, and drug abuse. Interactions between norepinephrine and dopamine at catecholamine transporters have been described [4,24], and the heterologous sensitization data presented herein suggest an additional level of interaction between dopamine and norepinephrine systems. Furthermore, a norepinephrine transporter-deficient mouse model was recently shown to have increased sensitivity to psychostimulants and D_2-like receptor agonists [25]. Together these observations provide evidence that norepinephrine and drugs that modulate synaptic levels of norepinephrine are important regulators of dopamine receptor signaling.

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