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The tolerance property of human D3 dopamine receptor is determined by specific amino acid residues in the second cytoplasmic loop

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Abstract

The D2 and D3 dopamine receptor subtypes are structurally homologous and couple to the same signal transduction pathways. Nevertheless, their evolutionary conservation suggests that the two subtypes might exhibit unique signaling characteristics. We previously determined that D3 but not D2S dopamine receptor exhibits a tolerance property in which the D3 receptor-activated G-protein coupled inward rectifier potassium currents progressively decreases upon repeated agonist stimulation. In this paper, using AtT-20 neuroendocrine cells stably expressing either human D3 or D2S receptor, we show that the tolerance property is also observed in the D3 receptor-adenylyl cyclase and D3 receptor-mitogen-activated protein kinase signaling pathways. We have previously shown that the second cytoplasmic loop of D3 receptor is required for tolerance. Here, using site-directed mutagenesis, we identified the specific amino acids in the D3 second cytoplasmic loop involved in the tolerance property. The results show that substitution of a non-conserved cysteine residue at position 147 with positively-charged lysine or arginine residues abolishes tolerance. Interestingly, the cysteine 147 residue is embedded in a putative phosphorylation site adjacent to two serine residues. Mutation of these serine residues to alanine also attenuates tolerance. Taken together, these structural studies suggest a role for phosphorylation in D3 receptor tolerance property.

Keywords: Desensitization; Potassium channels; Adenylyl cyclase; MAP kinase; Signaling; Dyskinesia

1. Introduction

Dopamine receptors belong to the super family of seven-transmembrane G-protein-coupled receptors [1,2] and are classified based on sequence homology, pharmacology, and signal transduction properties into D1-like and D2-like dopamine receptor subtypes [3]. The D1-like receptors include D1 and D5 receptor subtypes and the D2-like receptors include D2, D3, and D4 receptor subtypes. D2 and D3 receptors modulate various signal transduction pathways via associated G-proteins [1]. Agonist-stimulation of D2 and D3 receptors inhibits adenylyl cyclase and decreases cAMP production via Gαi/o subunit [2]. Initial studies were not able to show an effective coupling of D3 to adenylyl cyclase [4]; however, subsequent studies showed that the D3 receptor specifically couples to adenylyl cyclase V isoform overexpressed in heterologous expression systems [5]. In mammalian cell lines, D2 and D3 receptors also couple to the mitogen-activated protein kinase (MAPK) pathway [6,7].

In the AtT-20 neuroendocrine cells, we have previously demonstrated that the stably-transfected human D3 receptor robustly couples to natively-expressed, endogenous adenylyl cyclase V [8] and GIRK channels [9,10]. By comparing the coupling of D2S (the short isoform of D2) and D3 dopamine receptors to endogenous GIRK channels in AtT-20 cells, we have also demonstrated that the human D3, but not the D2S dopamine receptor exhibits tolerance and slow response termination properties [11]. The D3 receptor tolerance property is defined as a progressive decrease in agonist-induced GIRK response upon repeated agonist stimulation. The slow response termination property of the D3 receptor describes the prolonged delay in termination of agonist-induced GIRK response after the removal of the agonist. Together the tolerance and slow response termination properties distinguish D3 and D2 receptor signaling, which could be potentially important for normal physiological

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function. Our objective here was to further characterize the tolerance property of the D3 receptor and determine if the tolerance property is exhibited by other D3 receptor signal transduction pathways. In addition, given that the second cytoplasmic loop of D3 receptor is involved in the tolerance property, we were interested in identifying the specific amino acid residues in the second cytoplasmic loop that determine the tolerance property.

In the present study we demonstrate that agonist-induced tolerance of the D3 receptor is also exhibited in adenylyl cyclase and MAPK signaling pathways. Our results show that substitution of the non-conserved cysteine residue at position 147 with positively-charged amino acid residues abolishes D3 receptor tolerance property. Mutation of adjacent S145 and S146 residues (present in a putative serine phosphorylation site) also attenuates tolerance, suggesting a role for phosphorylation in the D3 receptor tolerance property.

2. Materials and methods

2.1. Cell culture and transfection

AtT-20 mouse pituitary cells were grown in Ham's F10 medium with 5% fetal bovine serum (FBS), 10% heat-inactivated horse serum, 2 mM glutamine and 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA). AtT-20 cells stably expressing human D2S (AtT-D2S), D3 (AtT-D3) or Flag™-tagged D3 (AtT-Flag™ D3) dopamine receptor subtypes were maintained in the above F10 culture media supplemented with 500 μg/ml G418 (Invitrogen). AtT-20 cells stably expressing Flag™-tagged D3C147K construct were generated by transfecting parental AtT-20 cells with the recombinant Flag™-tagged D3C147K construct using DMRIE-C transfection reagent (Invitrogen, Carlsbad, CA). Forty-eight hours post-transfection, the selection process was initiated by treating transfected cells with 1 mg/ml of geneticin (G418; Invitrogen, Carlsbad, CA). Selection medium was exchanged every 2–3 days, and the cells were grown for 14 days. Selected clones resistant to G418 were assayed for the expression of Flag™-tagged D3C147K receptor with a Flag™ antibody (Sigma, St. Louis, MO) using Western blot analysis. We selected clones that expressed the Flag™-tagged D3C147K receptor protein at the same level as the AtT-Flag™ D3 cell line. The AtT-20 cell line expressing Flag™-tagged D3C147K receptor (AtT-Flag™D3C147K) was also maintained in F10 culture media supplemented with 500 μg/ml G418. Radioligand binding studies using [^{3}H] quinpirole showed that the B_{max} values for the AtT-Flag™D3 and AtT-Flag™D3C147K clonal cell lines used in this study were 1028±63 and 952±43 fmol/mg, respectively. The binding affinity (K_D) of quinpirole for the Flag™ tagged D3 and Flag™ tagged D3C147K receptors were 4.89±0.38 nM and 3.97±0.42 nM, respectively. We have previously reported that AtT-D2S cell line has a B_{max} value of 854±20 fmol/mg [8].

In some studies, parental AtT-20 cells were transiently-transfected with 1.0 μg of plasmid containing wild-type or mutant human D3 receptors and 0.2 μg of plasmid containing the enhanced green fluorescent cDNA (EGFP; Clontech, Palo Alto, CA) per well, using the DMRIE-C reagent (Invitrogen). EGFP plasmid was used as a marker to identify transfected cells. For electrophysiological characterization, AtT-20 cells were plated onto glass coverslips coated with 40 μg/ml poly L-lysine (Sigma, St. Louis, MO) in a 12-well plate. All electrophysiological characterizations of transfected fluorescent cells were carried out 36–48 h post-transfection.

2.2. Construction of mutant receptors

The various human D3 dopamine receptor mutants were generated using a PCR-based subcloning strategy that utilized one primer that contained the required mutations and a flanking primer. The primer pair generated mutation containing amplicons with unique restriction sites. These restriction sites were used to replace the region in the wild type D3 receptor with the mutation containing amplicon. All mutant human D3 receptor plasmids were initially characterized by restriction enzyme analysis and finally by DNA sequencing. Generation and characterization of the Flag™ tagged human D3 receptor has been previously described [11]. The Flag™ tag was introduced at the N-terminal of the human D3 receptor [11]. The Flag™ tagged human D3C147K mutant was generated by subcloning and substituting a restriction fragment containing the C147K mutation into the wild type Flag™ tagged human D3 receptor. All plasmids encoding the wild type and mutant receptors were purified on two sequential cesium chloride density gradients. Before transfecting, all plasmids containing the mutations were subjected to DNA sequencing. The sequencing results showed that no extraneous mutations were introduced in these plasmids. All wild type and mutant receptor cDNA were subcloned into the pcDNA3.0 mammalian expression vector (Invitrogen).

2.3. Electrophysiology, drugs, and solutions

Agonist-activated currents were measured by the whole-cell patch clamp technique as described before [11]. Briefly, cells were held at −65 mV and inward K^+ currents induced by drug solutions were measured. The external solution (SES) used for K^+ current measurements was, in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES [pH 7.4], and 10 glucose and the pipette solution contained in mM: 130 K-Aspartate, 20 NaCl, 1 $MgCl_2$, 10 HEPES [pH 7.4], 10 glucose, 0.1 GTP, 5 Mg-ATP, and 1 EGTA. To enhance inwardly rectifying K^+ currents, controls and drug exposures were performed in solutions with elevated extracellular [K^+] (30 mM) by substitution for Na^+. (−) Quinpirole (Sigma, St. Louis, MO, USA) was dissolved in water and used at indicated concentrations. A 10-mM stock of dopamine (Sigma) was freshly dissolved in 100 mM ascorbic acid and used at a final concentration of 100 nM. Drug solutions were delivered to cells via a multi-barreled micropipette array. The current responses were normalized to the cell capacitance, to account for variation in cell size.

2.4. Data analyses

Currents were measured using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA) and sampled through a Digidata 1322A interface (Axon Instruments, Union City, CA, USA) using the pClamp 8.0 software (Axon Instruments, Union City, CA, USA). Data files were imported into SigmaPlot (SPSS Inc., Chicago, IL, USA) for analysis and display. SigmaPlot was also used to perform two-tailed Student's *t*-test. Analysis of variance (ANOVA) and the Holm's multiple pair-wise comparison tests were performed with Primer of Biostatistics software (Version 5.0, McGraw-Hill, New York, NY, USA). Data were considered statistically different when probability was <0.05.

2.5. Radioligand binding assay

We have previously described the procedure for membrane isolation and radioligand binding assay [8]. AtT20 clonal lines expressing human Flag™ tagged D3 and D3C147K receptors were grown in six 100-mm tissue culture plates to approximately 80% confluence. In experiments that evaluated persistent agonist binding, AtT-Flag™ D3 cells were pretreated with either SES or 100 nM non-radio labeled quinpirole for 1 min. Both control and pretreated cells were thoroughly washed several times in SES for a total of 20 min. The wash procedure included three quick rinses with 10 ml of SES, followed by four rinses with 10 ml SES. Each of the four rinses was for 5 min at room temperature (25 °C). To isolate membranes, cells were scraped in ice-cold Harvest Buffer (HB: 10 mM Tris–HCl [pH 7.4], 5 mM $MgSO_4$) and lysed with 10 strokes of a Dounce A homogenizer. The nuclei were pelleted at 1000×*g* for 5 min and the supernatant spun at 45,000×*g* for 20 min to pellet the membrane fraction. The membrane pellet was washed by resuspending the pellet in HB and repeating the high-speed centrifugation. All centrifugation steps were done at 4 °C. The washed membrane pellet was resuspended in Binding Buffer (BB: 50 mM Tris–HCl [pH 7.4], 120 mM NMDG, 5 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA) and stored at −70 °C.

Binding assays were performed as described previously [8]. The frozen membrane pellets were thawed on ice and resuspended using three short pulses

using a Branson sonifier. Membranes (20 to 75 μg) were incubated with various concentrations of [^{3}H]quinpirole (Specific activity-48.9 Ci/mmol; Perkin Elmer, Boston, MA, USA) in Binding Buffer (BB) for 60 min at room temperature (25 °C). Eticlopride (1 μM; Sigma, St. Louis, MO, USA) was used to define non-specific binding. Following incubation, the reactions were rapidly filtered through Whatman GF/B filters that were pretreated with 0.3% polyethylenimine (Sigma, St. Louis, MO, USA) and washed three times with ice-cold wash buffer (50 mM Tris–HCl, pH 7.4) using a cell harvester (Inotech Biosystems, Rockville, MD, USA). The filters were dried overnight, incubated in CytoScint™-ES scintillation cocktail (MP Biomedicals, Irvine, CA, USA) and radioactivity determined using a Beckman liquid scintillation counter (LS5000TA) with a counting efficiency of 59.7%. The binding assays were done in triplicates and each assay repeated two independent times. The data were analyzed using non-linear regression and the data points were fit with a one-site, two-parameter equation using Sigma Plot (SPSS Inc., Chicago, IL, USA).

2.6. Cyclic AMP assay

Cyclic AMP (cAMP) levels of pretreated and non-pretreated samples were measured using the cAMP Biotrak Enzymeimmunoassay (EIA) System (GE Healthcare, Piscataway, NJ). AtT-D2S, AtT-Flag™ D3 and AtT-Flag™D3C147K stably expressing human D2S, Flag™-tagged D3 and Flag™-tagged D3 C147K dopamine receptors, respectively, were plated in 12-well plates and cultured for 48 h. To minimize variations due to differences in cell plating density and growth rate, we used two wells for each variable. The pretreated samples received 100 nM quinpirole, while control samples without pretreatment received SES for 1 min. Both control and pretreated cells were thoroughly washed several times in SES for a total of 20 min. The wash procedure included three quick rinses with 1 ml of SES, followed by four rinses with 1 ml SES. Each of the four rinses was for 5 min at room temperature (25 °C). Following the washes, the cells were treated for 15 min at 37 °C with 10 μM forskolin+300 μM IBMX or 10 μM Forskolin+300 μM IBMX+100 nM quinpirole. Cells were lysed using the reagent provided in the kit. Sample lysates from the two wells were pooled, diluted 4-fold and 0.1 ml transferred into three independent wells of the 96-well EIA plate. The cAMP levels were measured by following the recommendations of the kit manufacturer (GE Healthcare). The final absorbance values were measured at 450 nm on a Model 680 Microplate Reader (Bio-Rad, Hercules, CA). The amount of cAMP in each well was determined by comparing the absorbance values of the treated variables with a series of standards provided with the kit. For each experiment, the standards were assayed in parallel and used to generate a standard curve with a linear detection range that spanned 13–3200 femtomol/well. The 4-fold dilution of the treated samples ensured that the cAMP levels were within the linear range of the cAMP assay kit. The cAMP levels in each treated sample were assayed in triplicate and adjusted to the total protein concentration in each sample. The entire experiment was repeated three independent times.

2.7. Detection of ERK phosphorylation

For the MAPK studies, AtT-D2S, AtT-Flag™ D3 and AtT-Flag™D3C147K cell lines were grown in 6-well plates until 80–90% confluent. The cells were incubated in Opti-MEM serum-free medium for 4 h prior to treatment. The cells were then pretreated with 200 nM QP or SES for 1 min, followed by several thorough SES washes for 20 min. Both control and pretreated cells were thoroughly washed several times in SES for a total of 20 min. The wash procedure included three quick rinses with 3 ml of SES, followed by four rinses with 3 ml SES. Each of the four rinses was done for 5 min at room temperature (25 °C). Subsequently, the cells were treated with 200 nM QP for 0, 2, or 5 min and lysed using a lysis reagent (62.5 mM Tris–HCl [pH 6.8], 10 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 200 mM DTT, 1 μg/ml phosphatase inhibitor (Sigma), 1 μg/ml protease inhibitor cocktail (Sigma), 2% SDS, 0.5% TritonX-100, and 10% glycerol). Following centrifugal clarification, the supernatants were boiled for 3 min at 95 °C and 30 μl of cell lysate loaded on a 10% SDS polyacrylamide gel. The separated proteins were electroblotted onto nitrocellulose membrane and blocked in 5% nonfat milk. Activated (phosphorylated) ERK1 and ERK2 were first detected using monoclonal phospho-p44/p42 MAPK antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA). Following the detection of phosphorylated proteins, the membrane was stripped and reprobed to detect total ERK1/ERK2 proteins. Total ERK proteins were detected using a polyclonal antibody (1:1000 dilution; Cell Signaling Technology) overnight at 4 °C. Horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibodies (1:2,000, Pierce Biochemicals, Rockford, IL) and SuperSignal® West Dura extended duration substrate chemiluminescence detection kit (Pierce Biochemicals, Rockford, IL) were used to detect the signals. Immunoblots shown are from representative experiments that were repeated at least three independent times with comparable results.

3. Results

3.1. D3 receptor exhibits tolerance property in three different signaling pathways

In AtT-20 neuroendocrine cells heterologously expressed human D3 and D2S receptors couple to endogenous GIRK channels [9,10] and endogenous adenylyl cyclase V [8]. We have previously shown that non-transfected AtT-20 cells do not express endogenous dopamine receptors and do not induce GIRK currents when treated with quinpirole [8, 9]. We have also shown that GIRK responses elicited by activating D3, but not D2S, dopamine receptors with synthetic agonist, quinpirole, exhibits tolerance [11]. To determine if the native dopamine receptor ligand also induces D3 receptor tolerance, we measured the GIRK response in AtT-20 cells stably expressing the human D3 receptor after multiple treatments of 100 nM dopamine. The results in Fig. 1A show that in AtT-20 cells stably expressing human D2S dopamine receptors (AtT-D2S), dopamine does not cause tolerance, inducing multiple responses of equal magnitude. In contrast, in AtT-20 cells stably expressing human D3 dopamine receptors (AtT-D3), dopamine induces tolerance with the magnitude of GIRK response progressively decreasing upon repeated dopamine application (Fig. 1B). Cumulative data suggest that dopamine, like quinpirole induces significant tolerance in D3 but not D2S dopamine receptor (Fig. 1C). The second D3 receptor-induced GIRK response elicited by dopamine was 60% less than the first (Fig. 1C). Given the equivalent effects of dopamine and quinpirole, in subsequent experiments we exclusively used the D2/D3 agonist quinpirole.

We next determined if the second D3 receptor-induced GIRK response could be increased by treating the cells with a higher concentration of agonist. We treated the AtT-D3 cell with 100 nM quinpirole, elicited the first GIRK response, washed out the agonist till the current returned to baseline and then treated the cell a second time with different concentrations of quinpirole (10, 30, 100, 300 and 1000 nM). The ratio of the second GIRK response to first GIRK response in this experiment was found to be 0.31±0.08 (10 nM, n=4 cells); 0.28±0.05 (30 nM, n=4 cells); 0.39±0.033 (100 nM, n=9 cells); 0.41±0.03 (300 nM, n=6 cells); and 0.40±0.06 (1000 nM, n=4 cells). These results suggest that the D3 receptor tolerance induced by the initial 100 nM quinpirole treatment is not overcome by treating the cells, a second time, with a higher concentration of quinpirole.

To determine the duration of tolerance and to simulate treatment conditions used in the biochemical experiments described later, we pretreated the AtT-D3 cells with 1 ml SES or 100 nM quinpirole for 1 min and washed the cells for 20 min

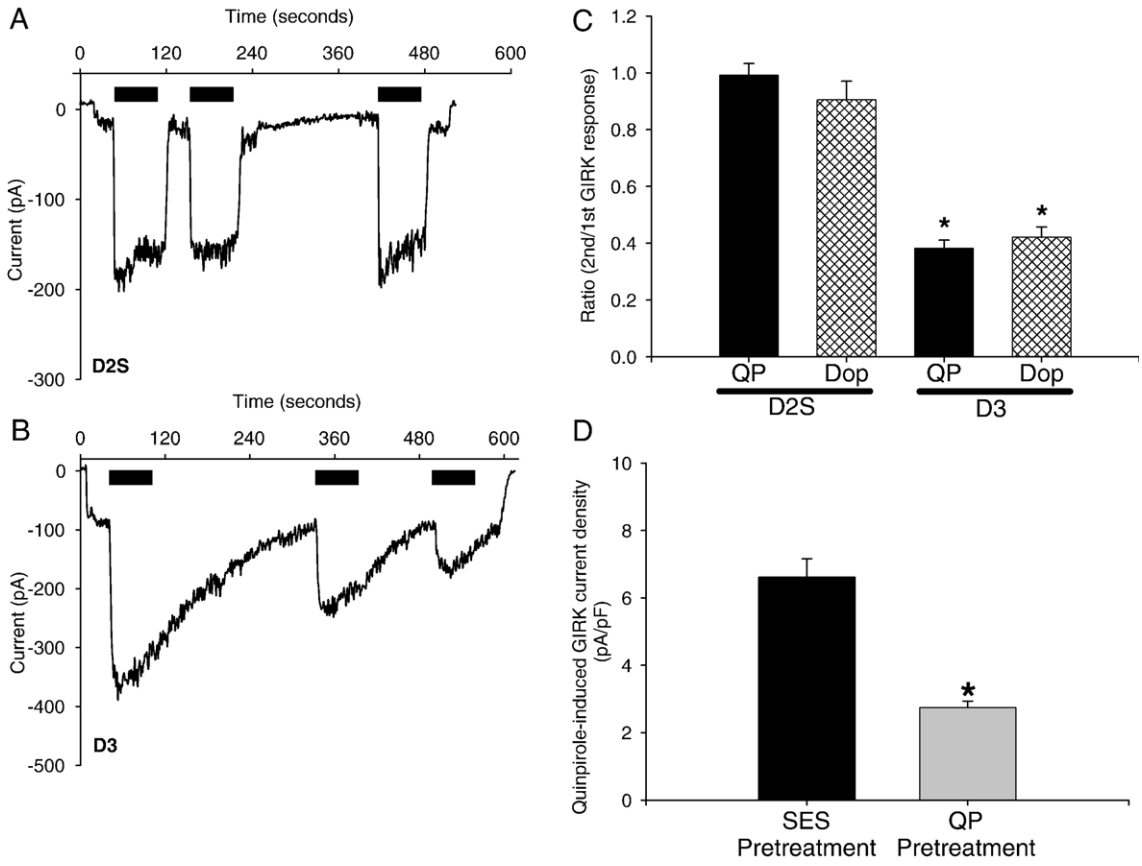

Fig. 1. Dopamine and quinpirole induce equivalent tolerance. Representative current traces from whole cell voltage clamp recording of AtT-D2S (A) or AtT-D3 (B) cell. The cell was held at −65 mV and inward currents were elicited by multiple 1 min applications of 100 nM dopamine (black rectangles). (C) Cumulative data showing the mean (±SE) ratio of second to first inward GIRK currents (tolerance) induced by either 100 nM quinpirole (QP, black bar) or 100 nM dopamine (Dop, hatched bar). The tolerance induced by both quinpirole and dopamine in AtT-D3 cells is significantly different from AtT-D2S cells (*$P<0.001$, $n=8$ cells, Student's *t*-test). (D) Cumulative data showing the mean (±SE) GIRK response elicited by 100 nM quinpirole in AtT-D3 cells that were pretreated for 1 min with either SES or 100 nM quinpirole and washed for 20 min as described in Materials and methods. The acute GIRK currents were normalized to cell capacitance. AtT-D3 cells pretreated with 100 nM quinpirole elicited a significantly smaller GIRK response compared to those pretreated with SES (*$P<0.001$, $n=9$ cells, Student's *t*-test).

as described in Materials and methods. Following the wash, whole cell voltage clamp recording was performed to measure 100 nM quinpirole-induced GIRK currents. The results in Fig. 1D show that the AtT-D3 cells pretreated with 100 nM quinpirole elicited a GIRK response (2.74±0.2 pA/pF) that was 58% less compared to cells pretreated with control SES (6.61±0.54 pA/pF). These results suggest that the D3 receptor-GIRK channel signaling pathway maintains its tolerance property even after a 20-min wash period.

In AtT-D2S and AtT-Flag™ D3 cells, following stimulation with 100 nM quinpirole, forskolin-induced cAMP level is inhibited approximately 50% (103.5±6.8 to 51.6±2.0 pmol/mg) and 59% (86.3±2.5 to 35.7±2.5 pmol/mg), respectively (Fig. 2A). To determine if the D3 receptor-adenylyl cyclase signaling pathway exhibits the tolerance property, we pretreated cells with either control SES or 100 nM quinpirole for 1 min. Following the extensive wash procedure described in Materials and methods, all cells were treated with 10 μM forskolin and 300 μM IBMX in the presence or absence of 100 nM quinpirole. Our results indicate that in AtT-Flag™ D3 cells pretreated for 1 min with 100 nM quinpirole, the ability of the second quinpirole treatment to inhibit forskolin-induced cAMP levels is significantly attenuated. Fig. 2A shows that in contrast to the 59% inhibition of forskolin-induced cAMP levels in control AtT-Flag™ D3 cells (86.3±2.5 to 35.7±2.5 pmol/mg), cells pretreated with 100 nM quinpirole showed only 28% inhibition (81.2±3.3 to 58.6±3.9 pmol/mg). The ability of D2S receptors to inhibit forskolin-induced cAMP levels was not affected by the 100-nM quinpirole pretreatment. The ratio of inhibitory response elicited in cells pretreated with quinpirole divided by the inhibitory response elicited in cells pretreated with control SES, gives a measure of tolerance. Fig. 2B shows that D3, but not D2S, receptor exhibits tolerance in the adenylyl cyclase signaling pathway.

Previous studies have shown that stimulation of D2 and D3 dopamine receptors induces activation of the MAPK pathway, with the maximum phosphorylation of p44 (ERK1) and p42 (ERK2) occurring at 5 min [6,7]. Consistent with these observations, we show that quinpirole treatment of AtT-D2S and AtT-Flag™ D3 cells induces robust phosphorylation of ERK1/ERK2 at 5 min (Fig. 3). To determine if the tolerance property of the D3 receptor is exhibited in the MAPK pathway we pretreated AtT-D2S or AtT-Flag™ D3 cells with either control SES or 200 nM quinpirole for 1 min. Following the

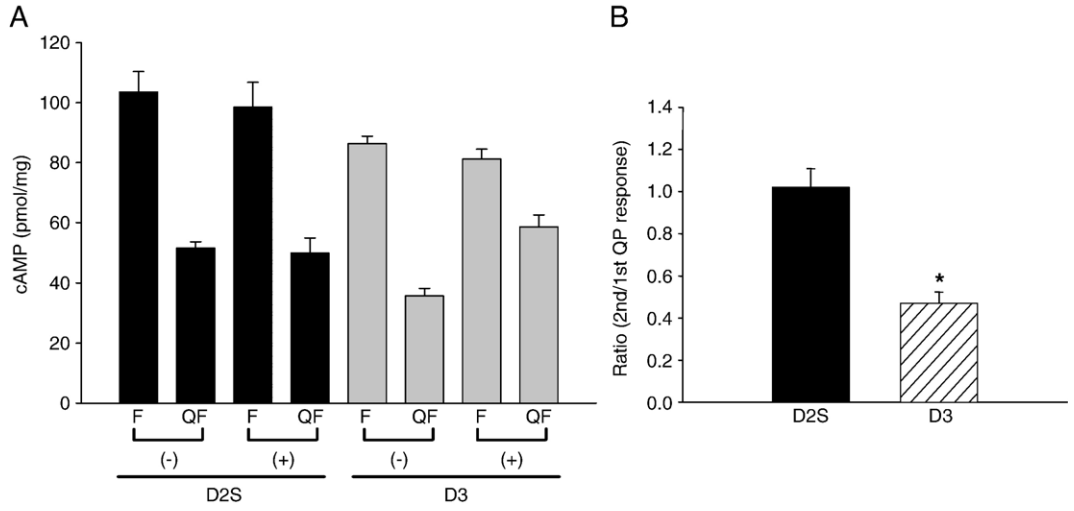

Fig. 2. Tolerance is exhibited in the D3-adenylyl cyclase signaling pathway. (A) Cumulative data showing the mean intracellular cAMP levels in AtT-D2S and AtT-Flag™ D3 cells treated with 10 μM forskolin and 300 μM IBMX in the absence (F) or presence of 100 nM quinpirole (QF). Before treating the cells with F or QF, the AtT-D2S and AtT-Flag™ D3 cells were pretreated for 1 min with either control SES (−) or 100 nM quinpirole (+) and washed for 20 min as described in Materials and methods. Under all conditions, in both cell lines, quinpirole significantly reduced the forskolin-induced increase in intracellular cAMP levels ($P<0.001$, Student's *t*-test). (B) However, mean tolerance, defined as the quinpirole-induced inhibition in cells that were pretreated with 100 nM quinpirole divided by quinpirole-induced inhibition in cells pretreated with control SES was significantly different between AtT-Flag™ D3 cells and AtT-D2S cells (*$P<0.001$, Student's *t*-test). AtT-Flag™ D3 cells pretreated with 100 nM quinpirole showed significantly reduced adenylyl cyclase inhibition upon second quinpirole treatment compared to cells pretreated with SES. The experiments were repeated three independent times.

extensive wash procedure described in Materials and methods, all cells were treated with or without 200 nM quinpirole for 2 and 5 min to induce ERK1/ERK2 phosphorylation. Fig. 3A shows that in AtT-D2S cells, phosphorylation of ERK1/ERK2 induced by the second quinpirole application was not significantly affected by the quinpirole pretreatment. In contrast, in AtT-Flag™ D3 cells, pretreatment significantly reduced the ERK1/ERK2 phosphorylation induced by the second quinpirole application (Fig. 3B). Measurement of the ratio of ERK phosphorylation induced with and without pretreatment show

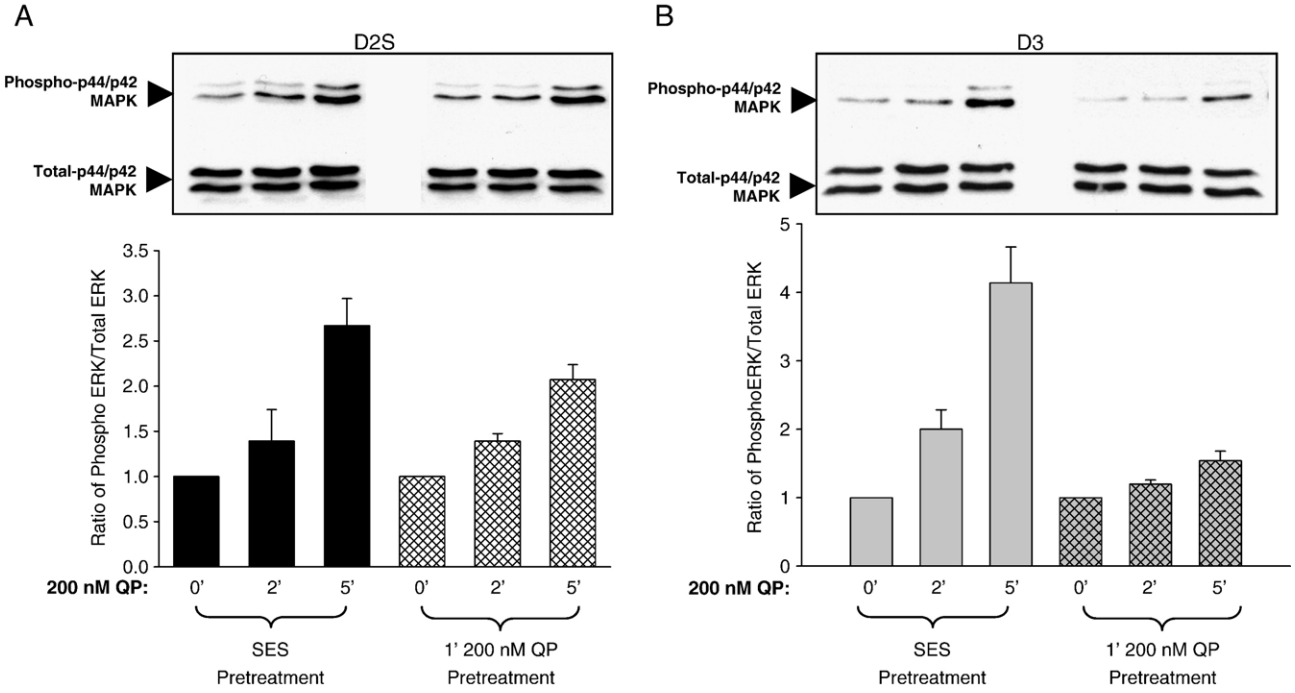

Fig. 3. Tolerance is exhibited in the D3-MAPK signaling pathway. Representative Western blots showing 200 nM quinpirole-induced phosphorylation of ERK1 and ERK2 (Phospho-p44/p42 MAPK) proteins in AtT-D2S (A) and AtT-Flag™ D3 (B) cells. Cells were pretreated for 1 min with either control SES or 200 nM quinpirole, washed and subsequently treated with control (0′) or 200 nM quinpirole for 2 and 5 min. The levels of quinpirole-induced phosphorylated MAPK proteins were normalized to the levels of total MAPK proteins. The experiments were repeated three independent times.

that D3 receptors (ratio = 0.45 ± 0.06) exhibit significant tolerance compared to D2S receptors (ratio = 0.80 ± 0.08).

Taken together, the results in Figs. 1–3 clearly demonstrate that the tolerance property of D3 receptor is exhibited by three different signal transduction pathways, and strongly suggest that tolerance is an intrinsic property of the D3 receptor that is not limited to a particular signaling pathway.

3.2. D3 receptor tolerance is not mediated by persistent agonist binding

Despite the extensive washes, the tolerance property of D3 receptor could be due to persistent agonist binding. To directly determine this, we performed radioligand binding assays with [^{3}H]quinpirole on AtT-Flag™ D3 cells that were pretreated for 1 min with control SES or 100 nM non-radiolabelled quinpirole and washed extensively as described in Materials and methods. If the non-radiolabelled quinpirole was persistently bound to the D3 receptor, the ability of the [^{3}H]quinpirole to bind to the cells would be reduced and reflected in the B_{max} and K_D values. The results in Fig. 4 show that neither B_{max} nor K_D values were altered by the 100-nM quinpirole pretreatment (B_{max} = 1028 ± 63 and 1112 ± 98 fmol/mg; K_D = 4.89 ± 0.38 nM and 4.11 ± 0.27 nM for control and pretreated cells, respectively). These results suggest that persistent binding of quinpirole to the D3 receptor does not induce tolerance. This is also consistent with the observations that in the three signaling pathways studied, the functional response recovers to the baseline following agonist washout.

3.3. Cysteine 147 in the second loop of the D3 receptor is involved in tolerance

We have previously determined that the second cytoplasmic loop of the D3 dopamine receptor is required for the tolerance property [11]. Since D2S dopamine receptors do not exhibit tolerance (Figs. 1–3 and [11]), we hypothesized that the

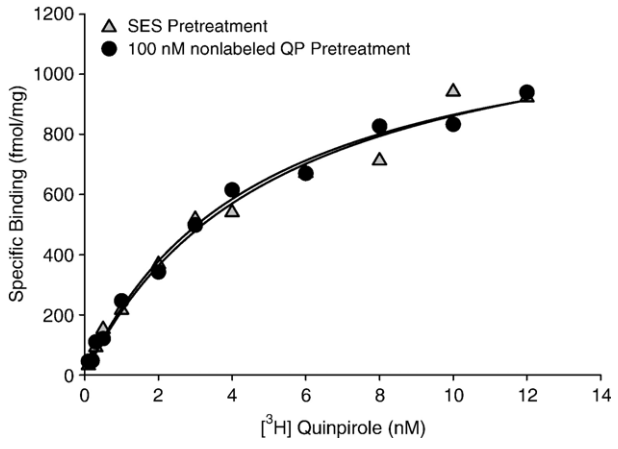

Fig. 4. Tolerance is not mediated by persistent quinpirole binding. Specific [^{3}H]quinpirole binding to AtT-Flag™ D3 cells that were pretreated for 1 min with either SES (grey upright triangle) or 100 nM non-labeled quinpirole (black filled circle) and washed for 20 min as described in Materials and methods. The saturation binding curves were obtained by fitting the data to a one-site model as described in Materials and methods and represent the mean of two independent experiments.

tolerance property of D3 receptor is mediated by amino acid residues in the second intracellular loop that are different from the D2S receptor (Fig. 5A). The D2S second cytoplasmic loop is also two amino acids shorter than the D3 receptor's loop, suggesting an alternate hypothesis that the difference in length of the second cytoplasmic loop between D2S and D3 receptor might be important for tolerance. To test the first hypothesis, we generated mutant D3 receptors in which the non-conserved amino acid residues in the second intracellular loop of the D3 receptor were mutated to the corresponding amino acid residues in the D2S receptor (D3GQ143RY and D3C147K). To test the second hypothesis, we generated a D3 receptor mutant (D3QHΔ) in which we deleted Q139 and H140 residues present in the second cytoplasmic loop of D3 receptor. As a control, to evaluate the functional importance of the Q139 and H140 residues, we also generated mutant D3 receptors in which either one (D3H140G) or both the Q139 and H140 residues were mutated to glycines (D3QH139GG).

The mutant and wild type D3 receptor expressing plasmids were transiently-transfected individually into AtT-20 cells. We assessed the function of mutant and wild type D3 receptors by measuring the quinpirole-induced GIRK responses using whole-cell, voltage-clamp recording method. All D3 receptor mutants were functional and induced a robust acute GIRK response (Table 1). The mean acute GIRK response induced by the wild-type D3 receptor and the mutant D3 receptors were similar with the exception of the QHΔ and QH139GG mutants. These mutant receptors showed a significantly reduced acute response than the wild-type D3 receptor (Table 1). To assess the tolerance property of the mutated D3 receptors, we determined the ratio of 2nd to 1st quinpirole-induced GIRK responses. The tolerance exhibited by the D3 receptor mutants was compared to the D2S and D3 wild-type receptors as well as the D3D2SII chimera (in which the entire second cytoplasmic loop of D3 receptor is replaced with the D2S receptor second cytoplasmic loop;). The cumulative data in Fig. 5B show that of all the mutants only D3C147K abolished the tolerance, exhibiting a mean tolerance value that was similar to D2S and D3D2SII chimeric receptors. These results strongly suggest that the cysteine 147 residue is centrally involved in the D3 receptor tolerance property. The results also suggest that a difference in the length of the second cytoplasmic loop between D2S and D3 receptor does not affect the tolerance property.

The loss of tolerance observed in the D3C147K mutant could be due to a decrease in receptor binding affinity for quinpirole or due to altered functional dose response. To address these issues, we compared the receptor binding affinity of D3 and D3C147K mutants using the clonal cell lines stably expressing the Flag™ tagged D3 receptor or the Flag™ tagged D3C147K mutant receptor. The binding affinity (K_D) of quinpirole for the Flag™ tagged D3 and Flag™ tagged D3C147K receptors were 4.89 ± 0.38 nM and 3.97 ± 0.42 nM, respectively. This result suggested that the loss of tolerance in the C147K mutant was not due to a decrease in binding affinity. To further confirm these results we performed a functional quinpirole dose response study comparing the ability of the Flag™ tagged D3 and D3C147K receptor to activate GIRK channels. A change in receptor binding affinity

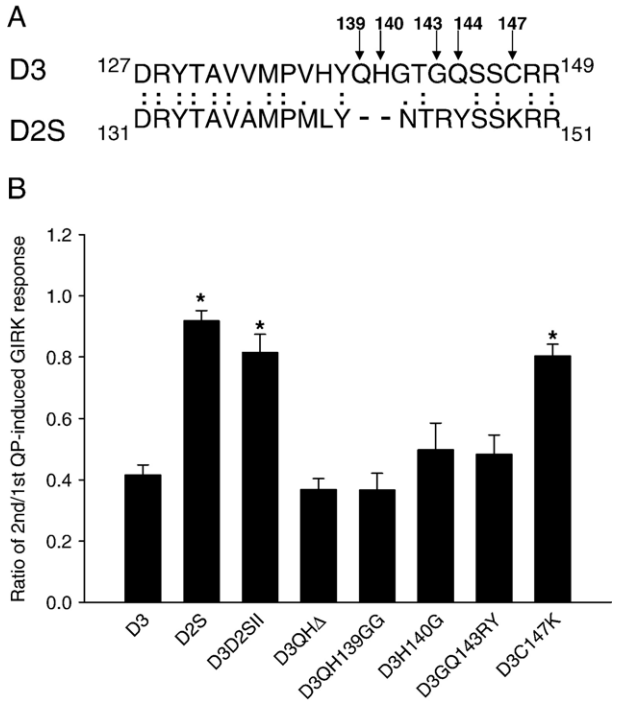

Fig. 5. Mutation of the C147 residue in D3 receptor abolishes tolerance. (A) Alignment of the second cytoplasmic loop of human D3 and D2S dopamine receptor shows the positions of non-conserved amino acids. The arrows indicate the amino acid residues that were mutated or deleted in this study. (B) Cumulative data showing the mean ratio of 2nd to 1st quinpirole (QP)-induced GIRK response in AtT-20 cells transiently-transfected with wild type D3, D2S, chimeric D3D2SII or various second cytoplasmic loop mutant D3 receptors. The GIRK responses were determined by measuring the inward currents elicited at −65 mV by 100 nM quinpirole in whole cell voltage clamp recording mode. The tolerance exhibited by D2S, D3D2SII and D3C147K receptors was significantly different from wild type D3 as well as the other second loop mutant receptors (*$P<0.01$, ANOVA, post-hoc Holm's test).

for quinpirole would be expected to shift the functional dose response curve. The results in Fig. 6 show that the wild type D3 and mutant D3C147K receptors have a similar dose response curve. The EC_{50} values for quinpirole-induced GIRK channel activation were 20.9±6.1 nM and 15.2±4.7 nM for Flag™ tagged D3 and Flag™ tagged D3C147K receptors, respectively. Together these results suggest that loss of tolerance in the D3C147K mutant is not due to changes in binding affinity.

To determine if the C147 residue of D3 receptor is also involved in the tolerance exhibited in the adenylyl cyclase and MAPK signaling pathways, we compared the ability of Flag™-tagged D3C147K to elicit multiple quinpirole-induced adenylyl cyclase inhibitory responses and ERK phosphorylation. Comparison of tolerance elicited in AtT-D2S, AtT-Flag™ D3 and AtT-Flag™ D3C147K cell lines shows that the tolerance property of D3C147K mutant receptor is significantly different from the D3 receptor and similar to the D2S receptor in both the adenylyl cyclase (Fig. 7A) and MAPK (Fig. 7B) signaling pathways. Taken together these results demonstrate that substitution of a single non-conserved cysteine residue at position 147 of D3 receptor with the corresponding lysine residue from D2S dopamine receptor abolishes the tolerance property in all three signaling pathways.

Table 1
Acute quinpirole-induced GIRK responses of wild type D2S, D3 and second cytoplasmic loop mutant D3 receptors

Name	Mean quinpirole-induced current density (±SE)	
	(pA/pF)	*N*
D3	6.1 (±0.50)	12
D3D2SII	9.1 (±0.77)[a]	13
D3QH Δ	4.1 (±0.45)[b]	8
D3QH139GG	3.7 (±0.59)[b]	11
D3H140G	5.1 (±0.64)	10
D3GQ143RY	5.9 (±0.87)	11
D3C147K	6.3 (±0.60)	9

Wild type and mutant D3 receptor constructs were transiently-transfected in to AtT-20 cells. Forty-eight hours post-transfection, whole cell voltage clamp recording was performed on individual cells. Cells were stimulated with 100 nM quinpirole and acute inward GIRK currents were measured and divided by cell capacitance to obtain current density (pA/pF). *N* indicates the number of cells tested.

[a] Significantly greater than the current density elicited with wild type D3 receptors ($P<0.005$, Student's *t*-test).

[b] Significantly less than the current density elicited with wild type D3 receptors ($P<0.01$, Student's *t*-test).

3.4. Positively charged amino acids at position 147 abolishes the tolerance property

The lack of tolerance exhibited by the D3C147K mutant suggests that intrinsic characteristics of the cysteine residue in D3 receptor might contribute to the tolerance property. Alternatively, the lack of tolerance could be attributed to intrinsic characteristic (such as charge or size) of the introduced lysine residue. To distinguish between these hypotheses, we generated several D3 receptor mutants in which the C147 amino acid residue was mutated to alanine (D3C147A), serine (D3C147S), glutamate (D3C147E) and arginine (D3C147R). Mutation of cysteine to alanine or serine introduces small, non-polar or nucleophilic amino acid residues at position 147 and

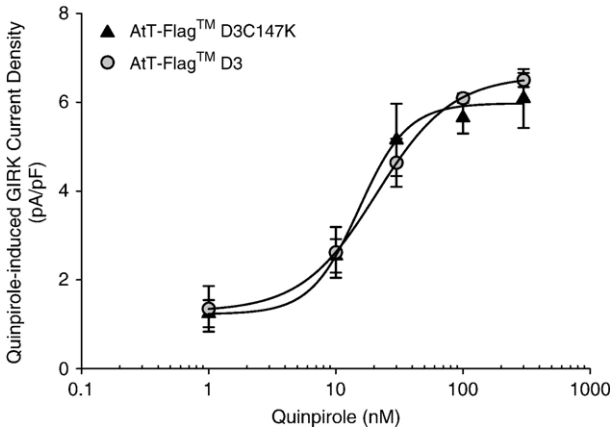

Fig. 6. Mutation of C147 residue in D3 receptor does not alter the quinpirole dose response curve. Dose-dependent increase in quinpirole-induced GIRK currents in AtT-Flag™ D3 (grey circle) and AtT-Flag™ D3C147K (black upright triangle) cells are similar. For each quinpirole concentration, we tested 5 and 6 cells for AtT-Flag™ D3 and AtT-Flag™ D3C147K cell lines, respectively. The data points were fit using a Hill 4 parameter equation.

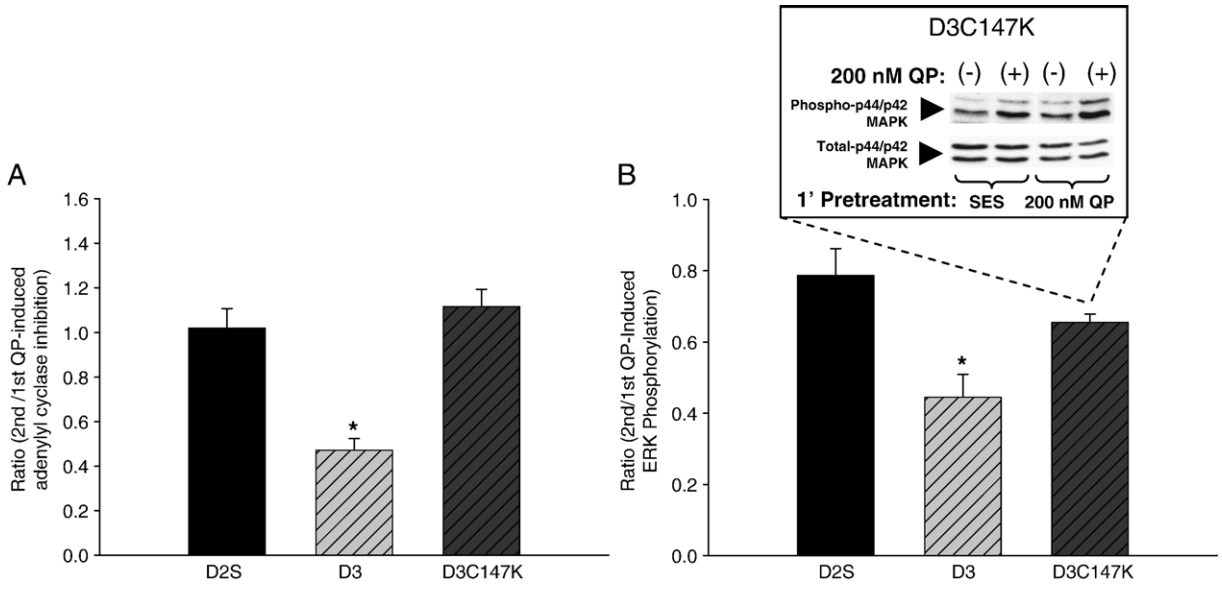

Fig. 7. Mutation of the C147 residue in D3 receptor abolishes tolerance property exhibited in D3-adenylyl cyclase and D3-MAPK signaling pathways. (A) Cumulative data comparing the mean tolerance of adenylyl cyclase inhibition in AtT-D2S, AtT-Flag™ D3 and AtT-Flag™ D3C147K cells. Tolerance was determined by dividing the 100-nM quinpirole-induced percent inhibition of forskolin-elevated cAMP levels in cells pretreated with 100 nM quinpirole by the 100-nM quinpirole-induced percent inhibition of forskolin-elevated cAMP levels in cells pretreated with control SES. The AtT-Flag™ D3 cells exhibit significantly reduced response following quinpirole pretreatment compared to both AtT-D2S and AtT-Flag™ D3C147K cells (*$P<0.001$, ANOVA, post-hoc Holm's test). (B) Cumulative data comparing the mean tolerance of MAPK activation in AtT-D2S, AtT-Flag™ D3 and AtT-Flag™ D3C147K cells. Tolerance was determined by dividing the 200-nM quinpirole-induced phosphorylation of ERK1/2 at 5 min in cells pretreated with 200 nM quinpirole by the 200-nM quinpirole-induced phosphorylation of ERK1/2 at 5 min in cells pretreated with control SES. The AtT-Flag™ D3 cells exhibits significantly reduced response following quinpirole pretreatment compared to both AtT-D2S and AtT-Flag™ D3C147K cells (*$P<0.05$, ANOVA, post-hoc Holm's test). The inset shows a representative Western blot showing 200 nM quinpirole-induced phosphorylation of ERK1 and ERK2 (Phospho-p44/p42 MAPK) proteins in AtT-Flag™ D3C147K cells. Cells were pretreated for 1 min with either control SES or 200 nM quinpirole, washed and subsequently treated with control (0′) or 200 nM quinpirole for 5 min. The levels of quinpirole-induced phosphorylated MAPK proteins were normalized to the levels of total MAPK proteins. The experiments were repeated three independent times.

would determine if intrinsic characteristics of the cysteine residue are important for tolerance. Mutation of cysteine to arginine or glutamate introduces positive and negative charges, respectively, at position 147 and would directly determine if charge or size of the lysine residue is responsible for abolishing tolerance.

The mutants and wild type D3 receptors were transiently-transfected individually into AtT-20 cells and the coupling of the receptors to endogenous GIRK channels was assessed using whole cell voltage clamp recording. The results in Table 2 show that all mutants were functional and elicited robust quinpirole-induced GIRK responses. However, comparison of mean acute quinpirole-induced GIRK responses showed that the D3C147E mutant elicited a significantly reduced GIRK response compared to wild type D3 receptors and other mutants (Table 2). Assessment of tolerance property measured by the ratio of second and first quinpirole-induced GIRK response showed that while D3C147A, D3C147S and D3C147E exhibited tolerance, the D3C147R mutant like the D3C147K mutant did not show tolerance (Fig. 8). These results strongly suggest that the intrinsic properties of the cysteine residue at position 147 do not contribute to the D3 receptor tolerance property. Rather, introduction of a positive charge (either lysine or arginine) at this position abolishes the tolerance property of the D3 receptor. Furthermore, a negative charge at position 147 is inhibitory and decreases the acute response induced by D3 receptor activation.

3.5. Mutation of a putative serine phosphorylation site flanking the C147 residue attenuates D3 receptor tolerance property

The results in Fig. 8 suggested that charged amino acid residues at position 147 modulate the tolerance property. Interestingly, the C147 residue is embedded in a putative phosphorylation site adjacent to serine residues (Fig. 9A). The

Table 2
Acute quinpirole-induced GIRK responses of wild type D3 and C147 mutant D3 receptors

Name	Mean quinpirole-induced current density (±SE)	
	(pA/pF)	*N*
D3	6.1 (±0.50)	12
D3C147K	6.3 (±0.60)	9
D3C147A	5.7 (±0.79)	9
D3C147S	5.8 (±0.85)	11
D3C147E	3.7 (±0.54)[a]	7
D3C147R	6.6 (±0.75)	8

Wild type and mutant D3 receptor constructs were transiently-transfected in to AtT-20 cells. Forty-eight hours post-transfection, whole cell voltage clamp recording was performed on individual cells. Cells were stimulated with 100 nM quinpirole and acute inward GIRK currents were measured and divided by cell capacitance to obtain current density (pA/pF). *N* indicates the number of cells tested.

[a] Significantly less than the current density elicited with wild type D3 receptors ($P<0.01$, Student's *t*-test).

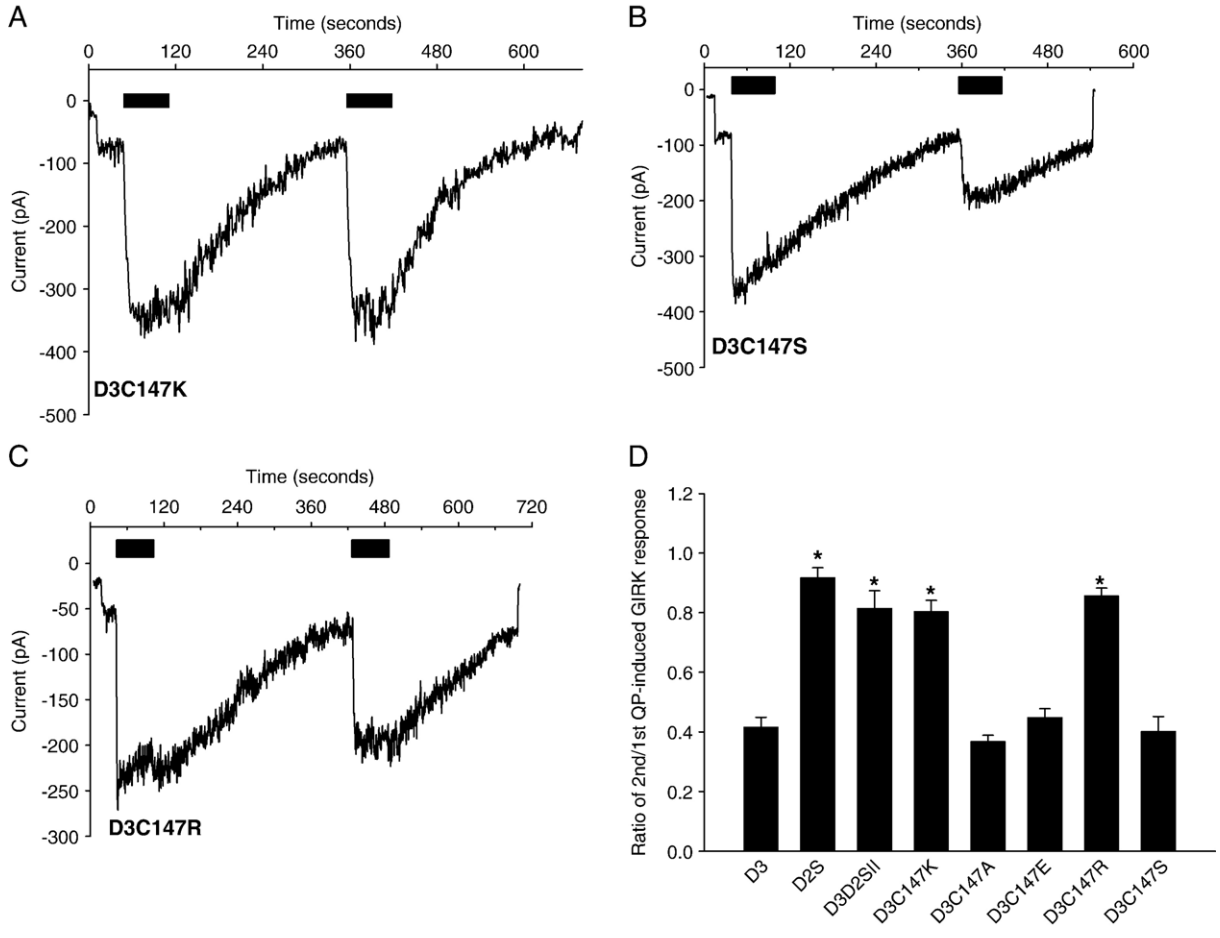

Fig. 8. Positively charged residues at position 147 abolish tolerance. (A) Representative current traces from whole cell voltage clamp recordings of AtT-20 cells transiently transfected with D3C147K (A), D3C147S (B) and D3C147R (C) mutants. The cells were held at −65 mV and inward currents were elicited by multiple 1 min applications of 100 nM quinpirole (black rectangles). (D) Cumulative data showing the mean ratio of 2nd to 1st quinpirole (QP)-induced GIRK response in AtT-20 cells transiently-transfected with wild type D3, D2S, chimeric D3D2SII or various second cytoplasmic loop mutant D3 receptors. The GIRK responses were determined by measuring the inward currents elicited at −65 mV by 100 nM quinpirole in whole cell voltage clamp recording mode. The tolerance exhibited by D2S, D3D2SII, D3C147K and D3C147R mutant receptors was significantly different from wild type D3 as well as the other second loop mutant receptors (*$P<0.01$, ANOVA, post-hoc Holm's test).

results in Fig. 8 suggested a hypothesis that phosphorylation might be involved in D3 receptor tolerance property, and introduction of a positively charged arginine or lysine residue adjacent to phosphorylatable serine residues might neutralize the effect of phosphorylation and abolish tolerance property. To test this hypothesis we generated mutant D3 receptors in which one (D3S146A) or both (D3SS145AA) serine residues were mutated to alanine and assessed for function and tolerance property. The acute quinpirole-induced GIRK response elicited by the D3S146A (5.6±0.67 pA/pF) and D3SS145AA (4.93±0.40 pA/pF) was similar to wild type D3 receptor (6.1±0.46 pA/pF). However, compared to wild type D3 receptors, both mutant receptors showed significantly attenuated tolerance (Fig. 9B). Interestingly, while the D3S146A and D3S145AA receptors both showed significantly reduced tolerance, unlike the D3C147K mutant, the tolerance was not completely abolished. Taken together these results suggest that the putative serine phosphorylation site in the second cytoplasmic loop of D3 receptor is partially involved in the tolerance property.

4. Discussion

The signaling mechanisms and properties of D3 dopamine receptor are not well understood. In this paper, we present novel evidence that the human D3, but not D2S dopamine receptor exhibits a tolerance property that affects three different signal transduction pathways. Structure–function analysis using site-directed mutagenesis of amino acid residues in the second cytoplasmic loop identified specific amino acid residues in a putative serine phosphorylation site involved in the tolerance property. Along with our previous study [11], the results presented in this paper strongly suggest that the tolerance property functionally distinguishes the D3 and D2 dopamine receptors. Our results show that the tolerance property is induced by both the endogenous ligand dopamine as well as the synthetic ligand quinpirole. It is an intrinsic property of the D3 receptor as it is observed in the D3-GIRK, D3-adenylyl cyclase and D3-MAPK pathways. A previous study, using the *Xenopus* oocyte heterologous expression system, has also reported the D3 receptor tolerance property [12]. We have observed the D3

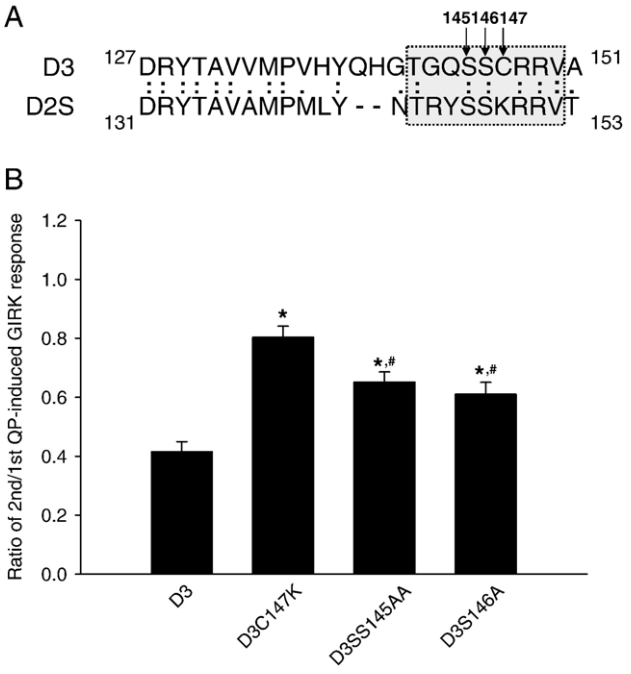

Fig. 9. Mutation of D3 receptor second cytoplasmic loop serine residues attenuates tolerance property. (A) Alignment of the second cytoplasmic loop of human D3 and D2S dopamine receptor shows the positions of non-conserved amino acids and a putative serine phosphorylation site (shaded box). The arrows indicate the serine residues that were mutated to alanine. (B) Cumulative data showing the mean ratio of 2nd to 1st quinpirole (QP)-induced GIRK response in AtT-20 cells transiently-transfected with wild type D3, D3C147K, D3SS145AA or D3S146A mutant receptors. The GIRK response was determined by measuring the inward currents elicited at −65 mV by 100 nM quinpirole in whole cell voltage clamp recording mode. The tolerance exhibited by D3C147K, D3SS145AA and D3S146A mutant receptors was significantly different from wild type D3 (*$P<0.01$, ANOVA, post-hoc Holm's test). In addition, D3SS145AA and D3S146A mutant receptors was significantly different from D3C147K mutant receptor ($^{\#}P<0.05$, ANOVA, post-hoc Holm's test).

receptor tolerance property when either the mouse or human D3 receptors and GIRK channels are heterologously expressed in AtT-20 or CHO cells (data not shown). The coupling of D3 receptor to signaling pathways in vivo has not been unambiguously determined; therefore, to date there have been no direct reports of D3 receptor tolerance property in vivo. Interestingly, in a study that characterized coupling of D2-like dopamine receptors to GIRK channels in primary neuronal cultures derived from rat substantia nigra pars compacta, the authors present data on two types of quinpirole-induced GIRK responses—one that does not show tolerance and one that shows robust tolerance [13]. We speculate that the former most likely represented coupling of D2 receptors to GIRK channels and the latter coupling of D3 receptors to GIRK channels.

Mutation or deletion of the Q139 and H140 residues reduced the acute quinpirole-induced GIRK response; however, mutation of H140 by itself had no effect. Together these results suggest that the Q139 residue in the D3 receptor second cytoplasmic loop is important for the function of the D3 receptor. At present, it is not known if the decreased function is due to alterations in receptor binding affinity, G-protein coupling or problems with protein folding, trafficking and cell surface expression. Regardless of its effects on acute response, the mutation of Q139 and/or H140 residues had no effect on D3 receptor tolerance. In contrast, the mutation of C147 residue to lysine or arginine completely abolished the tolerance property without affecting the acute response. Furthermore, the mutation of C147 residue to lysine did not change agonist binding affinity or shift the functional dose response curve. Interestingly, the introduction of a negatively charged residue at C147 also decreases the acute GIRK response without affecting tolerance. Taken together these results suggest that the tolerance property is not dependent on the magnitude of the acute D3 receptor-induced response.

Most G-protein coupled receptors (GPCRs) undergo desensitization in the continued presence of agonists [14]. In majority of these receptors, the desensitization is mediated by receptor phosphorylation, followed by internalization [14]. In the case of the D3 receptor, in the continued presence of the agonist, the receptor shows limited desensitization; however, once the agonist is removed the receptor manifests the tolerance property. While different from the desensitization property described for most GPCRs, the tolerance property of D3 receptor could be explained by phosphorylation and internalization mechanisms; however, we have previously demonstrated that the D3 receptor tolerance is not mediated by receptor internalization [11]. The mutational studies presented in this paper suggest that phosphorylation might be involved in the D3 tolerance property. Mutation of S146 residue in the second cytoplasmic loop of D3 receptor is sufficient to partly attenuate the tolerance property (Fig. 9). In addition, mutation of C147 residue to negatively charged glutamate residue decreases D3 receptor function (Table 2), a result that could be interpreted as the mutant receptor adopting a constitutive inhibitory conformation akin to the tolerance state. Our working hypothesis is that agonist binding to D3 receptor triggers a signaling pathway that activates protein kinase(s) which phosphorylates the S146 and potentially other residues. The phosphorylation induces a conformation change that uncouples the D3 receptor from its signaling pathway. We are currently investigating the signaling mechanisms and kinases involved in inducing D3 receptor tolerance.

There are several kinases that could potentially play a role in D3 receptor tolerance. In the case of the closely-related D2 receptor, the agonist-induced homologous desensitization is mediated by G-protein coupled receptor kinase (GRK) and β-arrestins [15,16]. An increased coupling of D2 and D3 receptors to their G proteins has been shown in GRK6 knockout mice, which display enhanced locomotor responses when challenged with dopamine agonists [17]. Thus, GRK-induced phosphorylation mechanisms involved in desensitization of various GPCRs [18,19] might also play a crucial role in agonist-induced tolerance of the D3 receptor. However, given that the D3 receptor does not undergo agonist-induced internalization during tolerance, any mechanism involving GRKs would be different from the traditional role of these kinases.

D2 receptor also shows heterologous desensitization when the receptor is phosphorylated by protein kinase C (PKC) [20]. The putative serine phosphorylation site in the second cytoplasmic

loop of the D3 receptor that is involved in the tolerance property is a consensus PKC site that is conserved between D2 and D3 receptors (Fig. 9). Interestingly, in D2 receptors this site in the second cytoplasmic loop is not phosphorylated by PKC [20]. Given the difference in the tolerance property between D2 and D3 receptor, we speculate that this site might be phosphorylated by PKC in the D3 receptor. We are currently testing this hypothesis.

The differences in tolerance property between D2 and D3 receptors have important functional consequences. In the context of GIRK channels and modulation of neuronal activity, the tolerance property of D3 receptor results in the generation of a firing pattern that is different from the D2 receptor. The D2 receptor hyperpolarizes the cell every time the receptors are stimulated by dopamine or synthetic agonists; in contrast, D3 receptors hyperpolarize the cell during the first stimulation but not during subsequent near-term stimulations [11]. Thus while both D2 and D3 receptors couple to GIRK channels to hyperpolarize the cell, the tolerance property of D3 receptor results in the modulation of the functional output, generating a different firing pattern. Increasing evidence indicates that the neuronal oscillations and coordination of ensemble activity plays an important role in the manifestation of motor behavior [21]. Thus the differences in tolerance property of D3 and D2 receptors might be important for normal physiological behavior. This role might be disrupted in pathophysiological conditions when the ratio of D3 and D2 receptor expression is altered during disease states or following chronic treatment. In particular, during levodopa-induced dyskinesia in Parkinson's disease the ratio of D2 to D3 receptor expression is significantly altered due to overexpression of D3 receptors in brain regions that predominantly express D2 receptors [22]. We propose that the alteration of D2 and D3 receptor expression ratio and the associated changes in the manifestation of tolerance property could significantly change coordinated firing patterns, generating asynchrony, and leading to the development of symptoms associated with dyskinesia.

Numerous studies have suggested a role for D3 receptor in stimulant drug induced behavioral sensitization [reviewed in 23]. It has been proposed that the loss of inhibitory D3 receptor function contributes to the development of behavioral sensitization [23]. While the D3 receptor tolerance property we describe develops rapidly, it might still be involved in the development of behavioral sensitization. In particular, if there are selective alterations in D3 receptor expression associated with stimulant exposure, we speculate that this change in D3 receptor expression might alter the homeostasis vis-à-vis the normal inhibitory response due to an over/under representation of the D3 receptor tolerance property. Thus, understanding the molecular mechanisms that cause tolerance might help address pathological conditions such as dyskinesia and addictive behaviors.

Acknowledgments

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