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Desensitization of the Dopamine D1 and D2 Receptor Hetero-Oligomer Mediated Calcium Signal by Agonist Occupancy of Either Receptor

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ABSTRACT

When dopamine D1 and D2 receptors were coactivated in D1-D2 receptor hetero-oligomeric complexes, a novel phospholipase C-mediated calcium signal was generated. In this report, desensitization of this $G_{q/11}$-mediated calcium signal was demonstrated by pretreatment with dopamine or with the D1-selective agonist (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF-81297) or the D2- selective agonist quinpirole. Desensitization of the calcium signal mediated by D1-D2 receptor hetero-oligomers was initiated by agonist occupancy of either receptor subtype even though the signal was generated only by occupancy of both receptors. The efficacy, potency, and rate of calcium signal desensitization by agonist occupancy of the D1 receptor ($t_{1/2}$, ~1 min) was far greater than by the D2 receptor ($t_{1/2}$, ~10 min). Desensitization of the calcium signal was not mediated by depletion of calcium stores or internalization of the hetero-oligomer and was not decreased by inhibiting second messenger-activated kinases. The involvement of G protein-coupled receptor kinases 2 or 3, but not 5 or 6, in the desensitization of the calcium signal was shown, occurring through a phosphorylation independent mechanism. Inhibition of G_i protein function associated with D2 receptors increased D1 receptor-mediated desensitization of the calcium signal, suggesting that cross-talk between the signals mediated by the activation of different G proteins controlled the efficacy of calcium signal desensitization. Together, these results demonstrate the desensitization of a signal mediated only by hetero-oligomerization of two G protein-coupled receptors that was initiated by agonist occupancy of either receptor within the hetero-oligomer, albeit with differences in desensitization profiles observed.

Dopamine receptors are members of the G protein-coupled receptor (GPCR) superfamily and play important roles in neuronal transmission and signal transduction. The five dopamine receptors, like most GPCRs, have been demonstrated to exist as homo-oligomers (George et al., 1998; Lee et al., 2000; O'Dowd et al., 2005) and hetero-oligomers (So et al., 2005). Hetero-oligomerization may account for some of the observed synergism between D1 and D2 receptors because D1 and D2 receptors have been demonstrated to be coexpressed in neurons (Surmeier et al., 1992; Aizman et al., 2000; Maltais et al., 2000). Our observations of D1 and D2 receptors occurring within the same signaling complexes were determined by coimmunoprecipitation from rat brain (Lee et al., 2004). In heterologous cells coexpressing both receptors, the existence of hetero-oligomers was established by fluorescence resonance energy transfer (So et al., 2005), cotrafficking studies (So et al., 2005), and visualization of D1-D2 hetero-oligomers in live cells (O'Dowd et al., 2005). Of greater physiological significance was the finding that activation of a novel phospholipase C-mediated calcium signal was observed only when both D1 and D2 receptors within

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; KN93, *N*-[2-[[[3-(4′-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4′-methoxybenzenesulfonamide phosphate salt; D4476, 4-(4-(2,3-dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1*H*-imidazol-2-yl)benzamide; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole; dbcAMP, dibutyryl-cAMP; PMA, phorbol 12-myristate 13-acetate; SCH 23390, (*R*)-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; SKF81297, (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide; ADPβS, adenosine-5′-0-(2-thiodiphosphate) trilithium salt; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; siRNA, small interfering RNA; Con A, concanavalin A; PKA, protein kinase A; PKC, protein kinase C; Bis, bisindolylmaleimide I; CAMKII, Ca^{2+}-calmodulin kinase II; AFU, absolute fluorescence units; PTX, pertussis toxin.

hetero-oligomeric complexes were coactivated but not when D1 and D2 receptors were expressed alone (Lee et al., 2004). These hetero-oligomeric complexes were observed in brain, resulting in novel activation of $G_{q/11}$ proteins (Rashid et al., 2007). The generation of this unique signal only by hetero-oligomerization between these two receptors provides an example of how diversity of receptor function can be achieved from a limited number of receptors and represents the first example within the rhodopsin family of GPCRs to elicit such an effect. The discovery of this dopamine-mediated calcium signal is important because it implies a direct link between dopamine receptors, generally considered to function largely by modulating adenylyl cyclase activation, and calcium generation. Because these D1-D2 receptor complexes are linked to calcium signaling, it is of major significance then to determine how this signal is regulated.

An important component of GPCR signaling is the termination of the signal by receptor desensitization. This process has been described to occur in vivo and is an important process by which neurons regulate and turn off signaling (Gainetdinov et al., 2004). All of the research on receptor desensitization, however, has been performed on signals generated by receptor homo-oligomers. Whether the mechanisms established for homo-oligomers are similar for hetero-oligomers remains to be elucidated.

In this study, we investigated the desensitization of the calcium signal associated with agonist activation of D1-D2 receptor hetero-oligomers. We provide evidence for rapid desensitization elicited by pretreatment with dopamine or with selective D1 or D2 receptor agonists. The efficacy, potency, and rate of signal desensitization differed between agonists that selectively occupied the D1 or D2 receptors or both receptors simultaneously. A role was shown for G protein-coupled receptor kinases (GRK) 2 or 3, but GRK5 and GRK6 receptor internalization, or any other second messenger-activated kinase, was not involved in the desensitization of the signal.

Materials and Methods

Measurement of the Calcium Signal. Stable cell lines coexpressing the amino-terminal hemagglutinin epitope-tagged human D1 receptor and amino-terminal FLAG epitope-tagged human D2 receptor were created in HEK293TSA cells using the pBudCE 4.1 vector (Invitrogen, Carlsbad, CA) and was characterized previously (Lee et al., 2004). All cell culture and transfection reagents were obtained from Invitrogen. HEK293TSA cells were maintained as monolayer cultures at 37°C in advanced minimum essential medium supplemented with 6% fetal bovine serum, 300 μg/ml Zeocin (Invitrogen), and antibiotics. Calcium mobilization assays were carried out using a FLEXstation multiwell plate fluorometer (Molecular Devices, Sunnyvale, CA). Stably transfected cells were seeded in black microtiter plates at a density of 10^5 cells/well and grown for 24 h. The cells were then loaded with 2 μM Fluo-4 acetoxymethyl ester indicator dye (Invitrogen) in advanced minimum essential medium supplemented with 2.5 mM probenecid (Sigma Aldrich, Oakville, ON, Canada) for 1 h and subsequently washed twice with Hanks' balanced salt solution without sodium bicarbonate and phenol red and supplemented with 20 mM HEPES (Invitrogen). Baseline fluorescence values were measured for 15 s, and changes in fluorescence corresponding to alterations in intracellular calcium levels upon the addition of agonists (indicated on each calcium tracing by a downward arrow) thereafter were recorded. Fluorescence values were collected at 3-s intervals for 100 s. For calculation of concentration response curves, the difference between maximum and minimum fluorescence values for each agonist concentration was determined and analyzed using Prism software (GraphPad, San Diego, CA). For desensitization studies, cells were pretreated with agonists in serum free advanced minimum essential medium for the time indicated and washed off with Hanks' balanced salt solution before calcium measurement. For inhibitor studies, cells were pretreated with 10 μM H89 (Sigma), 500 nM bisindolylmaleimide I, 10 μM KN93, 1 μM D4476, or 1 μM TBB (Calbiochem, San Diego, CA) 1 h before agonist pretreatment. To block receptor internalization, 250 μg/ml concanavalin A (Con A; Calbiochem) was coadministered with agonists tested. For protein kinase activator studies, cells were pretreated with 3 mM dibutyryl-cAMP (dbcAMP) (Calbiochem) for 15 min or with 1 μM phorbol 12-myristate 13-acetate (PMA; Calbiochem) for 30 min before calcium measurement. SKF81297, dopamine, quinpirole, SCH 23390, raclopride, ADPβS, EGTA, SQ22536, and pertussis toxin were purchased from Sigma. For studies involving G protein-coupled receptor kinases, transient transfections of cDNA encoding GRK2, GRK3, GRK5, or GRK6 in the mammalian expression vector pcDNA3 (Invitrogen) were performed with Lipofectamine (Invitrogen). The GRK constructs were a kind gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). YM254890 was kindly provided by Astellas Pharma Inc. (Tokyo, Japan).

Small Interfering RNA Silencing of Gene Expression. Chemically synthesized double-stranded siRNA duplexes (with 3′ dTdT overhangs) were purchased from Qiagen Inc. (Mississauga, ON, Canada) for GRK2 (5′-AAGAAGUACGAGAAGCUGAG-3′) and GRK3 (5′-AAGCAAGCUGUAGAACACGUA-3′) and were validated elsewhere (Violin et al., 2006). A nonsilencing RNA duplex (5′-UUCUCCGAACGUGUCACGU-3′) was used as a control for all siRNA experiments. Human embryonic kidney-293TSA cells were transfected with Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Silencing was quantified by immunoblotting.

Cell Surface Immunofluorometry. Cells stably expressing D1 and D2 receptors were plated in 96-well clear-bottomed plates (Corning Glassworks, Corning, NY) at a confluence of 1.5×10^5 cells per well 24 h before the experiment. Cells were treated for 30 min with agonist, fixed by 4% paraformaldehyde, blocked with 4% bovine serum albumin in phosphate-buffered saline, incubated with a 1:200 dilution of anti-HA (Roche) or a 1:1000 dilution of anti-FLAG (Invitrogen) antibodies for 1 h, washed with phosphate-buffered saline, and then labeled with a secondary antibody conjugated with fluorescein isothiocyanate (Invitrogen). Fluorescence was detected with a spectrophotometer (Cytofluor 4400; Applied Biosystems, Foster City, CA).

[^{3}H]cAMP Accumulation. [^{3}H]cAMP accumulation in cells stably expressing D1 receptors were determined as described previously (Hasbi et al., 1998). In brief, cells were seeded in 24-well plates at a density of 300,000 cells per well and incubated overnight in a culture medium supplemented with 0.6 μCi of [^{3}H]adenine (final volume, 300 μl). Cells were then washed once with DMEM/20 mM HEPES, pretreated with 1 μM dopamine for 30 min, and washed off. [^{3}H]cAMP accumulation was measured in the presence of 1 mM 3-isobutyl-1-methylxanthine and 10 μM dopamine for 5 min at 37°C. The reaction was stopped by addition of 250 μl of 5% trichloroacetic acid, and the separation of [^{3}H]cAMP was carried out by chromatography.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting. The procedures used for protein gel electrophoresis and immunoblotting were identical to those described previously (So et al., 2005). Rabbit anti-GRK2, -GRK3, -GRK5, and -GRK6 antibodies were obtained (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and used at a 1:200 dilution.

Results

To study calcium signal desensitization, a stable cell line coexpressing D1 and D2 receptors (Lee et al., 2004) (D1 =

2071 ± 60 fmol/mg; D2 = 2449 ± 90 fmol/mg) was pretreated for 30 min with either dopamine, the D1 receptor-selective agonist SKF81297, or the D2 receptor-selective agonist quinpirole. The ability of 1 μM dopamine, SKF81297, and quinpirole to activate the calcium signal is shown in Fig. 1A. The addition of dopamine stimulated a robust calcium signal that peaked within seconds of agonist activation and declined in less than 2 min. SKF81297 elicited a calcium signal that also peaked within seconds of agonist activation but achieved a peak value 50% of that elicited by dopamine. The addition of quinpirole did not elicit a calcium signal. Coadministration of SKF81297 and quinpirole generated a signal of the same magnitude as dopamine (data not shown). The calcium signal was $G_{q/11}$-protein linked, in that the addition of 10 nM $G_{q/11}$ inhibitor YM254890 (for structure, see Takasaki et al., 2004) completely abolished the dopamine-activated signal (Fig. 1B). The coactivation of D1 and D2 receptors mobilized calcium release from intracellular calcium stores because the signal was maintained in the presence of 250 μM EGTA, a calcium chelator (Fig. 1C). When cells were pretreated for 30 min with 1 μM dopamine and then washed off, the signal activated subsequently by 10 μM dopamine was attenuated compared with control (62.3 ± 3.7% of control, $n = 4$, $p < 0.05$) with similar kinetics of the calcium signal compared with control (Fig. 1D). To characterize this effect further, cells pretreated with 1 μM dopamine were activated with increasing concentrations of dopamine from 10^{-11} to 10^{-5} M. No significant change in the EC_{50} compared with control was observed in this concentration response experiment (control EC_{50} = 34.0 ± 4.1 nM, $n = 4$; treated EC_{50} = 39.4 ± 8.3 nM, $n = 4$, $p > 0.05$) (Fig. 1E) with a 30% decrease in maximal activation. To determine whether desensitization of the D1-D2 receptor-mediated signal could be achieved by heterologous desensitization resulting from the activation of another $G_{q/11}$-linked receptor, the endogenously expressed purinergic P2Y1 receptors were activated with agonist ADPβS at 1 μM for 30 min in D1-D2 receptor-expressing cells (Fig. 1F). The P2Y1 receptor calcium signal activated by 10 μM ADPβS was significantly desensitized by pretreatment with ADPβS, but no attenuation of the D1-D2 receptor-mediated

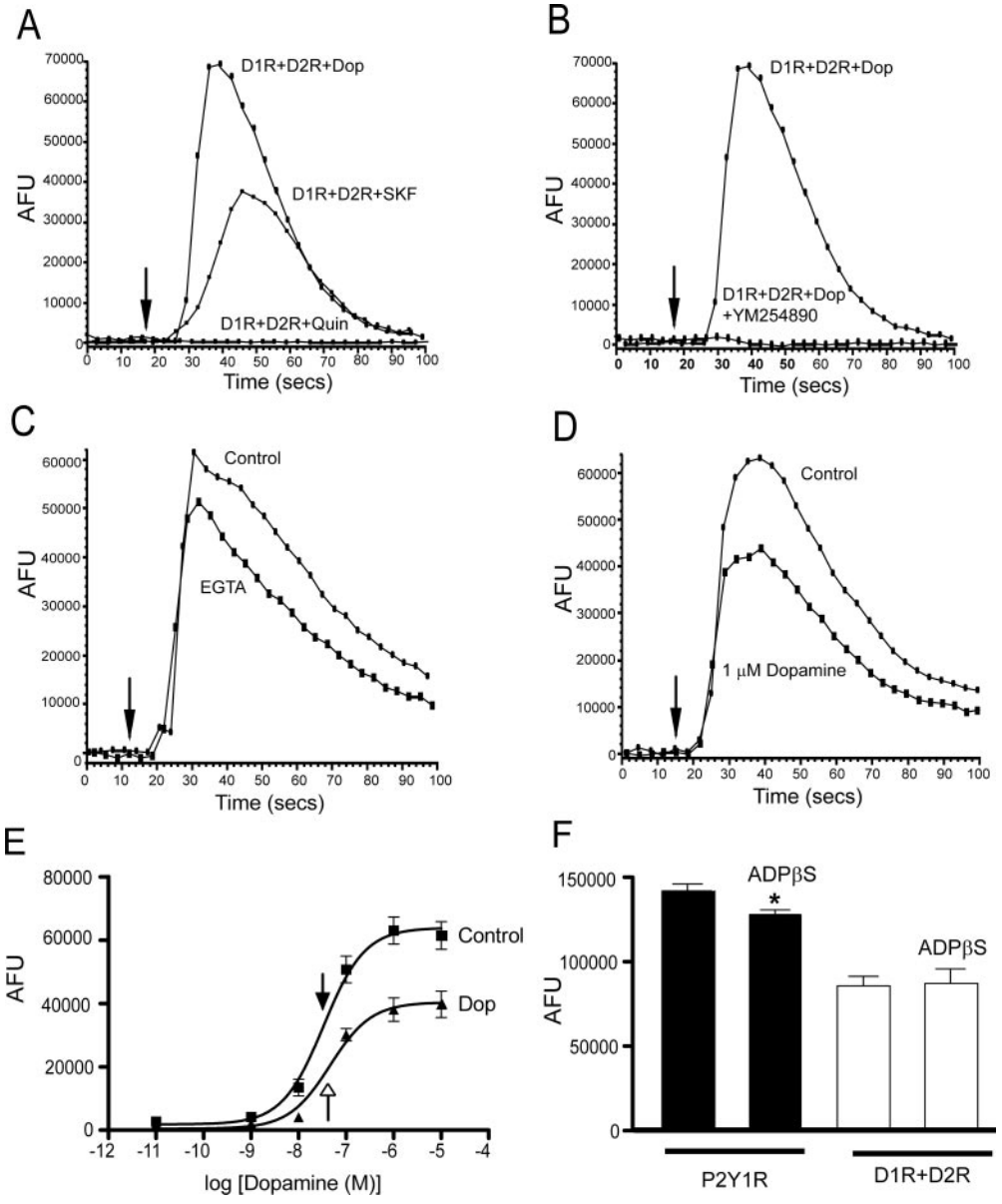

Fig. 1. The calcium signal associated with the coactivation of D1 and D2 receptors in cells stably expressing both receptors is desensitized by prior treatment with dopamine for 30 min. Data were expressed in AFU. A, the calcium signal from the addition of 1 μM dopamine, SKF81297, or quinpirole in cells stably expressing both receptors ($n = 5$). B, activation of the calcium signal by 1 μM dopamine is inhibited by 10 nM YM254890, a $G_{q/11}$ inhibitor ($n = 4$). C, activation of the calcium signal by 1 μM dopamine is not abolished by 250 μM EGTA, a calcium chelator ($n = 4$). D, the calcium signal activated by 10 μM dopamine (control) and after pretreatment with 1 μM dopamine for 30 min. E, effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μM dopamine treatment for 30 min ($n = 4$). Arrows indicate EC_{50} values. F, desensitization of the calcium signal associated with dopamine activation does not occur after the activation of the purinergic P2Y1 receptor with the selective agonist ADPβS ($n = 5$). Activation of the P2Y1 receptor by 10 μM ADPβS without (bar 1) or after 30-min pretreatment with 1 μM ADPβS (bar 2). Activation of the calcium signal by 10 μM dopamine (bar 3) or after 30-min pretreatment with 1 μM ADPβS (bar 4) (*, $p < 0.05$).

signal was observed after stimulation of P2Y1R, suggesting that the desensitization of the D1-D2 receptor complex occurred specifically by a homologous mechanism.

Because D1 and D2 receptor homo-oligomers have been previously demonstrated to desensitize in response to their receptor selective agonists (Ng et al., 1997; Lamey et al., 2002), we examined the effect of selective D1 or D2 receptor agonists on calcium signal desensitization (Fig. 2). Pretreatment for 30 min with 1 μM SKF81297 displayed desensitization that was significantly greater than that elicited by dopamine pretreatment (37.1 $\pm$ 7.1% of control, $n = 4$, $p < 0.05$) (Fig. 2A). Activating the signal after SKF81297 pretreatment with increasing concentrations of dopamine demonstrated no change in the EC_{50} of the signal compared with control (control EC_{50} = 34.0 $\pm$ 4.1 nM, $n = 4$; treated EC_{50} = 33.3 $\pm$ 11.3 nM, $n = 4$, $p > 0.05$) with a 60% decrease in maximal activation (Fig. 2B). Pretreatment with 1 μM quinpirole for 30 min elicited desensitization that was less than that obtained with dopamine (78.1 $\pm$ 3.5%, $n = 4$, $p < 0.05$) (Fig. 2C). Activating the signal after quinpirole pretreatment with increasing concentrations of dopamine demonstrated no change in the EC_{50} of the signal compared with control (control EC_{50} = 34.0 $\pm$ 4.1 nM, $n = 4$; treated EC_{50} = 38.0 $\pm$ 13.2 nM, $n = 4$. $p > 0.05$) with a 20% decrease in maximal activation (Fig. 2D). Persistent occupancy of the ligand binding pocket by dopamine, SKF81297, and quinpirole was not a concern, because an increase in the EC_{50} value would have been expected with continued ligand occupancy, but this was not observed. Because SKF81297 can act as a partial agonist for D2 receptors within the D1-D2 hetero-oligomeric complex (Rashid et al., 2007), the desensitization may be mediated by occupancy of both receptors. To prevent SKF81297 occupancy of the D2 receptor, 1 μM raclopride, a D2-selective antagonist, was added with SKF81297 pretreatment for 30 min (Fig. 2E). No significant difference in the extent of desensitization was observed. However, SKF81297-mediated desensitization was abolished by 10 μM SCH 23390, a D1 antagonist (Fig. 2E). This suggests that the desensitization elicited

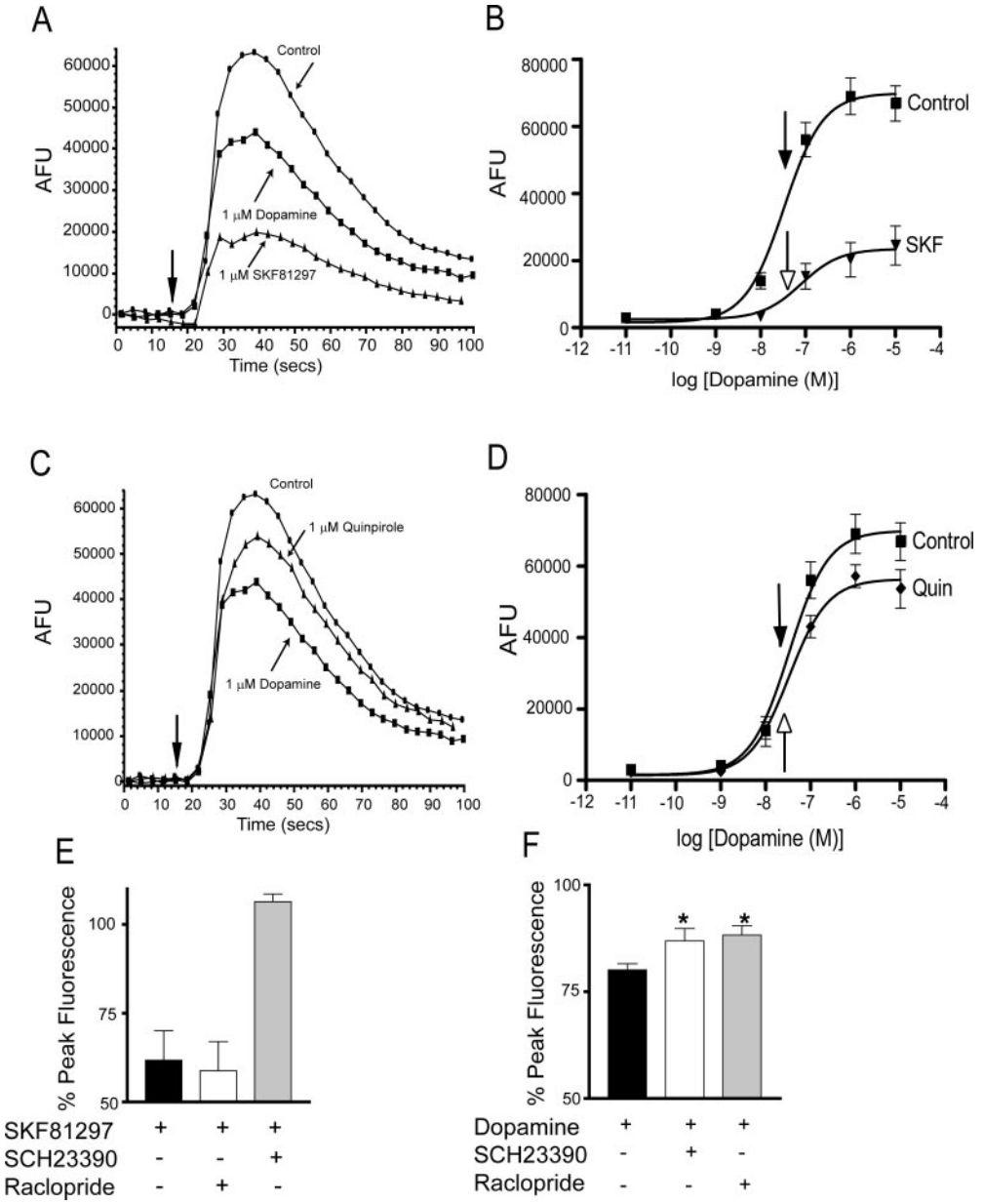

Fig. 2. The calcium signal associated with the coactivation of D1 and D2 receptors in cells stably expressing both receptors is desensitized by prior treatment with selective D1 or D2 agonists for 30 min. Data were expressed in absolute fluorescence units (AFU). A, the calcium signal activated by 10 μM dopamine (control) and after pretreatment with 1 μM SKF81297 for 30 min ($n = 3$). B, effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μM SKF81297 treatment for 30 min ($n = 4$). Arrows indicate EC_{50} values. C, the calcium signal activated by 10 μM dopamine (control) and after pretreatment with 1 μM quinpirole for 30 min ($n = 4$). D, effect of increasing concentrations of dopamine on the activation of the calcium signal without (control) and after 1 μM quinpirole treatment for 30 min ($n = 4$). Arrows indicate EC_{50} values. E, desensitization of the calcium signal elicited by pretreatment with 1 μM SKF81297 for 30 min without or with pretreatment with 10 μM SCH 23390 or 1 μM raclopride ($n = 4$). F, desensitization of the calcium signal elicited by pretreatment with 1 μM dopamine for 30 min without or with pretreatment with 1 μM SCH 23390 or 1 μM raclopride ($n = 4$).

by SKF81297 occurred through the selective occupancy of the D1 receptor. To determine whether dopamine is activating both receptors, D1-D2–expressing cells were cotreated with equivalent amounts of either SCH 23390 or raclopride. Fifty percent inhibition of dopamine-mediated desensitization was observed upon cotreatment with either antagonist (Fig. 2F).

Because the occupancy of either receptor within the heterooligomer mediated different extents of signal desensitization, we sought to determine whether this might also have an effect on the potency to desensitize the signal (Fig. 3A). Cells were pretreated for 30 min with increasing concentrations of dopamine, SKF81297, or quinpirole, from 10^{-11} to 10^{-5} M, washed, and activated with 10 μM dopamine. The D1 receptor-selective agonist SKF81297, through selective occupation of the D1 receptor, was very potent in desensitizing the signal, with an EC_{50} of 29.8 ± 8.4 nM (n = 5). Dopamine, which occupied both receptors, was less potent with an EC_{50} of 293.6 ± 83.2 nM (n = 5). Quinpirole, which occupied the D2 receptor, was the least potent of the agonists tested, with an EC_{50} of 1041 ± 160.8 nM (n = 5). The rank order of potency correlated with that for the extent of desensitization. Because the extent and potency of desensitization depended on the selective occupancy of either receptor, we investigated whether this correlation was also observed for the rate of signal desensitization (Fig. 3B). Cells were pretreated with 1 μM dopamine, SKF81297 or quinpirole from 5 to 60 min, washed and activated with 10 μM dopamine. SKF81297 desensitized the signal quickly with a $t_{1/2}$ of 0.94 ± 0.1 min (n = 4). Dopamine desensitized the signal more slowly than SKF81297, with a $t_{1/2}$ of 4.9 ± 1.1 min (n = 4). Quinpirole was the slowest desensitizer of the signal, with a $t_{1/2}$ of 9.5 ± 2.5 min (n = 4).

To determine the mechanism by which the calcium signal desensitized, we tested potential mediators of this effect. First, because depletion of intracellular calcium stores by prolonged agonist treatment is a mediator of desensitization of some $G_{q/11}$ protein coupled receptors (Yu and Hinkle, 1997), we sought to determine whether the amount of intracellular calcium remaining was a limiting factor contributing to lowered calcium responses by quantifying intracellular calcium release after agonist pretreatment. The sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) was added in the presence of the extracellular calcium chelator EGTA to quantify intracellular calcium levels in sarcoplasmic endoplasmic reticulum Ca^{2+}-ATPase–controlled stores after treatment with various agonists and compared with control levels. The amount of calcium released was not changed significantly by pretreatment with any of the agonists tested compared with control, suggesting that no calcium store depletion occurred (dopamine, then thapsigargin = 98.5 ± 6.2% of control; SKF81297, then thapsigargin = 108.7 ± 11.9% of control; quinpirole, then thapsigargin = 108.3 ± 3.5% of control). To verify this result, P2Y1 receptors, which used $G_{q/11}$ protein and phospholipase C as a means to generate calcium release through intracellular calcium stores (Schachter et al., 1996), were activated after

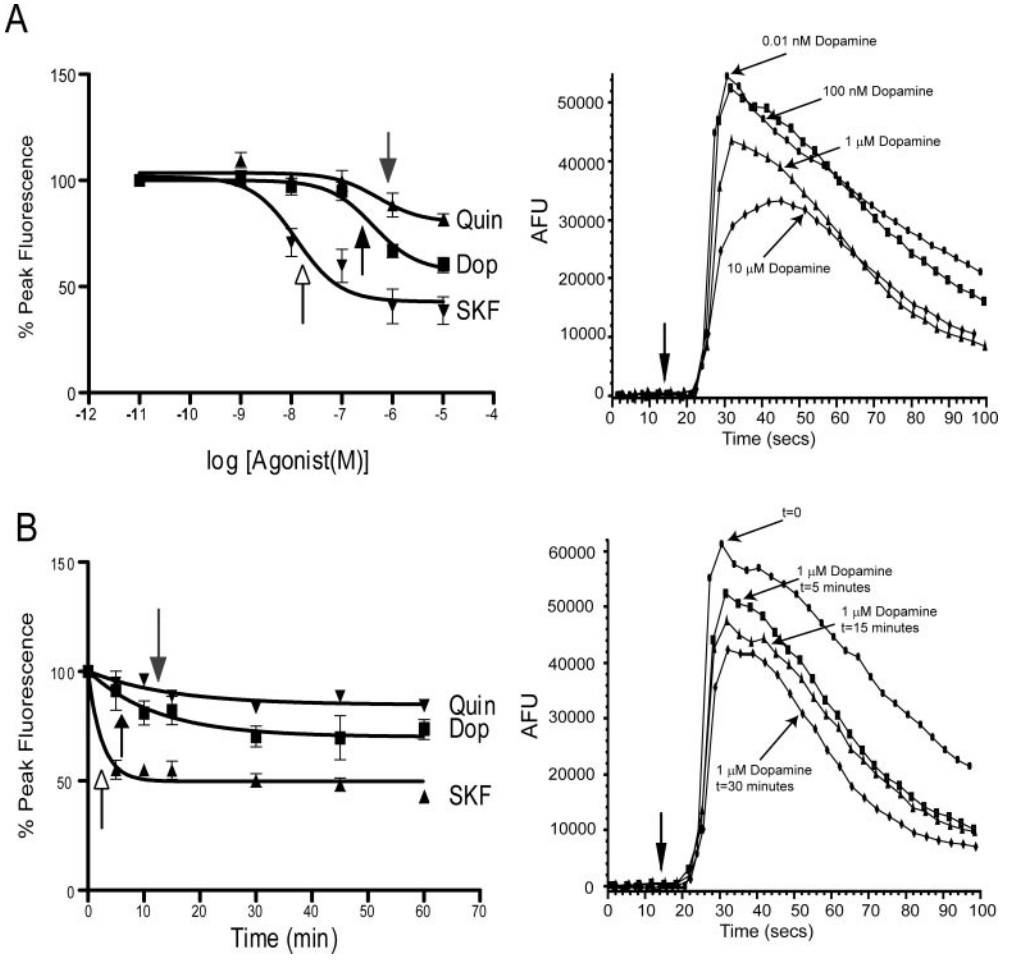

Fig. 3. The efficacy, potency, and rate of desensitization of the D1-D2–activated calcium signal varied among the agonists tested. Data were represented as the percentage of peak fluorescence of the calcium signal from 10^{-11} M dopamine-treated cells ± S.E.M. A, effect of increasing concentrations of agonists used during pretreatment on the desensitization of the calcium signal. Cells were pretreated with increasing concentrations of dopamine, SKF81297 or quinpirole, from 10^{-11} to 10^{-5} M for 30 min, washed, and activated with 10 μM dopamine. The rank order of drug potency mediating desensitization was SKF81297 > dopamine > quinpirole. Arrows indicate EC_{50} values (n = 5). Right, representative calcium tracing of the signal activated by 10 μM dopamine after pretreatment with increasing concentrations of dopamine for 30 min is shown. B, effect of increasing pretreatment times of agonists on calcium signal desensitization. Cells were pretreated with 1 μM dopamine, SKF81297, or quinpirole from 5 to 60 min, washed, and activated with 10 μM dopamine. The rank order of the drug effect mediating the rate of desensitization was SKF81297 > dopamine > quinpirole. Arrows indicate $t_{1/2}$ values in minutes (n = 5). Right, representative calcium tracing of the signal activated by 10 μM dopamine without pretreatment (t = 0 min) and after pretreatment with 1 μM dopamine from 5 to 30 min is shown.

dopaminergic agonist treatment in the presence of EGTA. No significant difference was observed, suggesting that calcium stores were not significantly depleted after agonist treatment (dopamine, then ADPβS = 91.9 ± 4.0% of control; SKF81297, then ADPβS = 104.6 ± 5.3% of control; quinpirole, then ADPβS = 92.9 ± 3.9% of control). Activation of P2Y1 receptors after dopaminergic agonist treatment was also performed in the absence of EGTA, and no significant differences were observed (data not shown). Because the calcium stores were not depleted, these results suggested that calcium signal desensitization might occur at the level of the receptor complex, either from the loss of receptor complexes from the cell surface or alteration of receptor complex function.

To evaluate the role of internalization in the desensitization mechanism, cells coexpressing D1 and D2 receptors were cotreated with 250 μg/ml Con A to keep the receptor complex at the cell surface, along with the chosen agonists for 30 min (Fig. 4). Cotreatment of agonists and Con A significantly attenuated agonist-induced receptor internalization of the D1 (Fig. 4A) and D2 receptors (Fig. 4B) in cells coexpressing both receptors as determined by quantitative cell surface immunofluorescence. Significantly attenuating receptor internalization did not result in a concomitant decrease in the extent of signal desensitization by the agonists tested; instead, a small, but significant, increase was observed (Fig. 4C).

Two of the major protein kinase families that have been implicated in desensitization of GPCRs are protein kinase A (PKA) and protein kinase C (PKC). The involvement of these protein kinases in D1 and D2 receptor-mediated desensitization when these receptors are expressed alone is well documented (Jiang and Sibley, 1999; Namkung and Sibley, 2004). Therefore, we tested whether the inhibition of these kinases by PKA and PKC inhibitors, 10 μM H89 and 500 nM bisindolylmaleimide I (Bis), respectively, attenuated desensitization mediated by dopamine (Fig. 5A), SKF81297 (Fig. 5B), and quinpirole (Fig. 5C). In all cases, the addition of neither the PKA nor the PKC inhibitor decreased the extent of desensitization. Pretreatment with the broad spectrum PKC inhibitor staurosporine alone or with Bis also did not inhibit signal desensitization (data not shown). It is noteworthy that inhibiting PKA activity increased desensitization mediated by all agonists tested. Representative calcium tracings for the effect of PKA and PKC inhibitors on dopamine-mediated desensitization are shown in Fig. 5, D and E. To test whether desensitization of the D1-D2 receptor-mediated signal can occur by heterologous mechanisms involving PKA and PKC, their respective activators, 3 mM dbcAMP and 1 μM PMA were used and were observed to decrease the calcium signal. The effects of these activators were attenuated significantly by H89 and Bis, respectively (Fig. 5F). These data suggest that these kinases did not have a direct role in homologous desensitization but may be able to indirectly desensitize the signal by heterologous activation of PKA and PKC.

GRKs have been implicated in the desensitization of D1 (Lamey et al., 2002) and D2 receptors (Ito et al., 1999) when expressed alone. Therefore, to explore these potential interactions, GRK2, -3, -5, and -6, reported to be endogenously expressed in HEK293T cells (Iwata et al., 2005), were transiently transfected into D1-D2 receptor-expressing cells. This strategy was previously used successfully to determine the effect of GRKs on the desensitization of dopamine receptors (Kim et al., 2001). Increased expression of GRK2 and GRK3

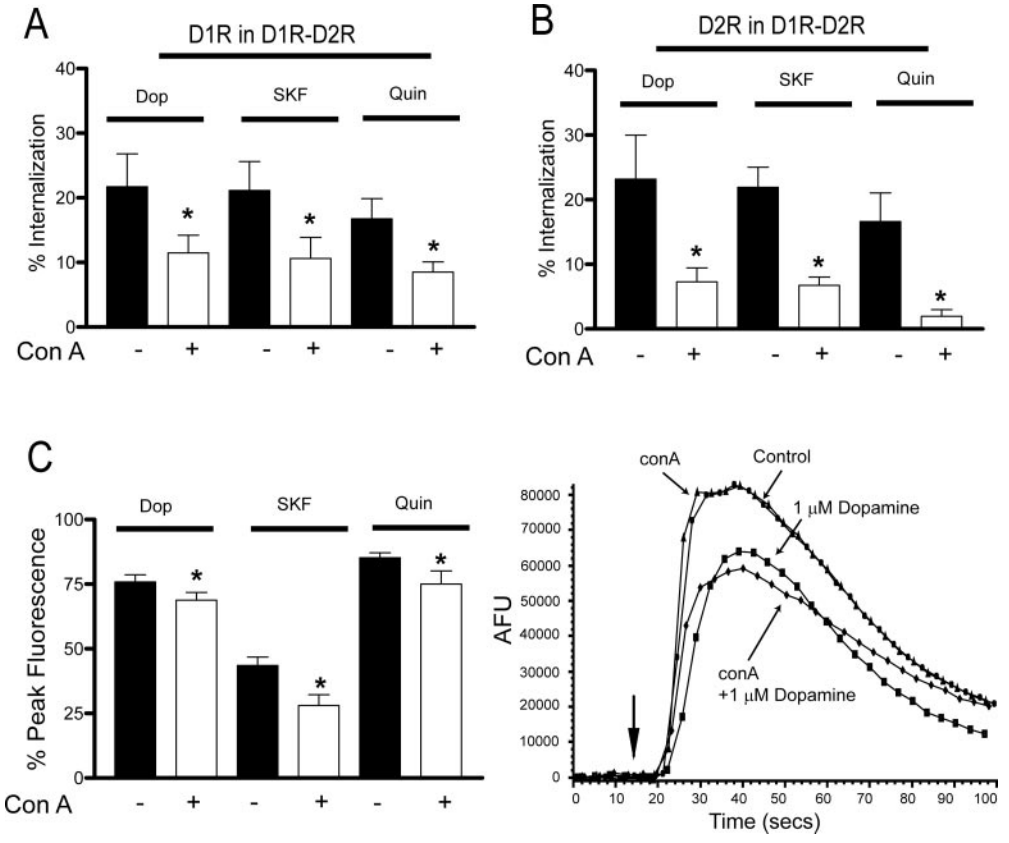

Fig. 4. Decreasing D1 and D2 receptor internalization in cells coexpressing both receptors with cotreatment of Con A and dopaminergic agonists enhanced agonist-mediated D1-D2–activated calcium signal desensitization. Internalization of the D1 receptor (A) or D2 receptor (B) in cells coexpressing D1 and D2 receptors treated for 30 min with 1 μM dopamine, SKF81297, or quinpirole in the absence (black bars) and presence (white bars) of 250 μg/ml Con A (n = 4–6). Percentage internalization represents the loss of fluorescence from the cell surface of intact cells after agonist treatment compared with vehicle treated controls. C, desensitization of the calcium signal after 30-min pretreatment with 1 μM dopamine, SKF81297, or quinpirole in the absence (black bars) and presence (white bars) of 250 μg/ml of Con A (n = 10). Right, representative calcium tracing for the effect of Con A on dopamine-mediated signal desensitization is shown. Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle pretreated cells ± S.E.M. (*, $p <$ 0.05).

increased desensitization significantly with all agonists tested (Fig. 6, A and B), whereas increasing the expression of GRK5 and GRK6 did not (Fig. 6, C and D), suggesting a role for GRK2 and -3 in signal desensitization. Increased expression of GRKs in transfected cells was confirmed by Western blot (Fig. 6E). A representative calcium tracing for the effect of increased GRK2 expression on dopamine-mediated desensitization is demonstrated in Fig. 6F.

To confirm the role of GRK2 and -3, we silenced endogenous GRKs with siRNA as described previously (Violin et al., 2006). Transfection of siRNA to silence GRK2 in D1-D2 receptor-expressing cells resulted in a significant decrease in the desensitization mediated by all agonists (Fig. 7A). Likewise, transfection of siRNA to silence GRK3 also significantly decreased the desensitization of the calcium signal mediated by all agonists (Fig. 7B). Decreased expression of these GRKs in transfected cells was confirmed by Western blot (Fig. 7, inset).

To elucidate a potential mechanism by which GRKs mediate desensitization, D1-D2 receptor-expressing cells were pretreated for 30 min with 200 μM zinc chloride, a general GRK inhibitor that inhibits the ability of GRKs to phosphorylate receptors (Benovic et al., 1987). Pretreatment with zinc chloride did not inhibit the desensitization of the calcium signal elicited by dopamine, SKF81297, or quinpirole pretreatment (Fig. 8A) but was successful in inhibiting the desensitization of the cAMP signal generated by the activation of the D1 receptor stably expressed in human embryonic kidney cells (2071 ± 60 fmol/mg) by a 30-min dopamine pretreatment (Fig. 8B). It is noteworthy that desensitization

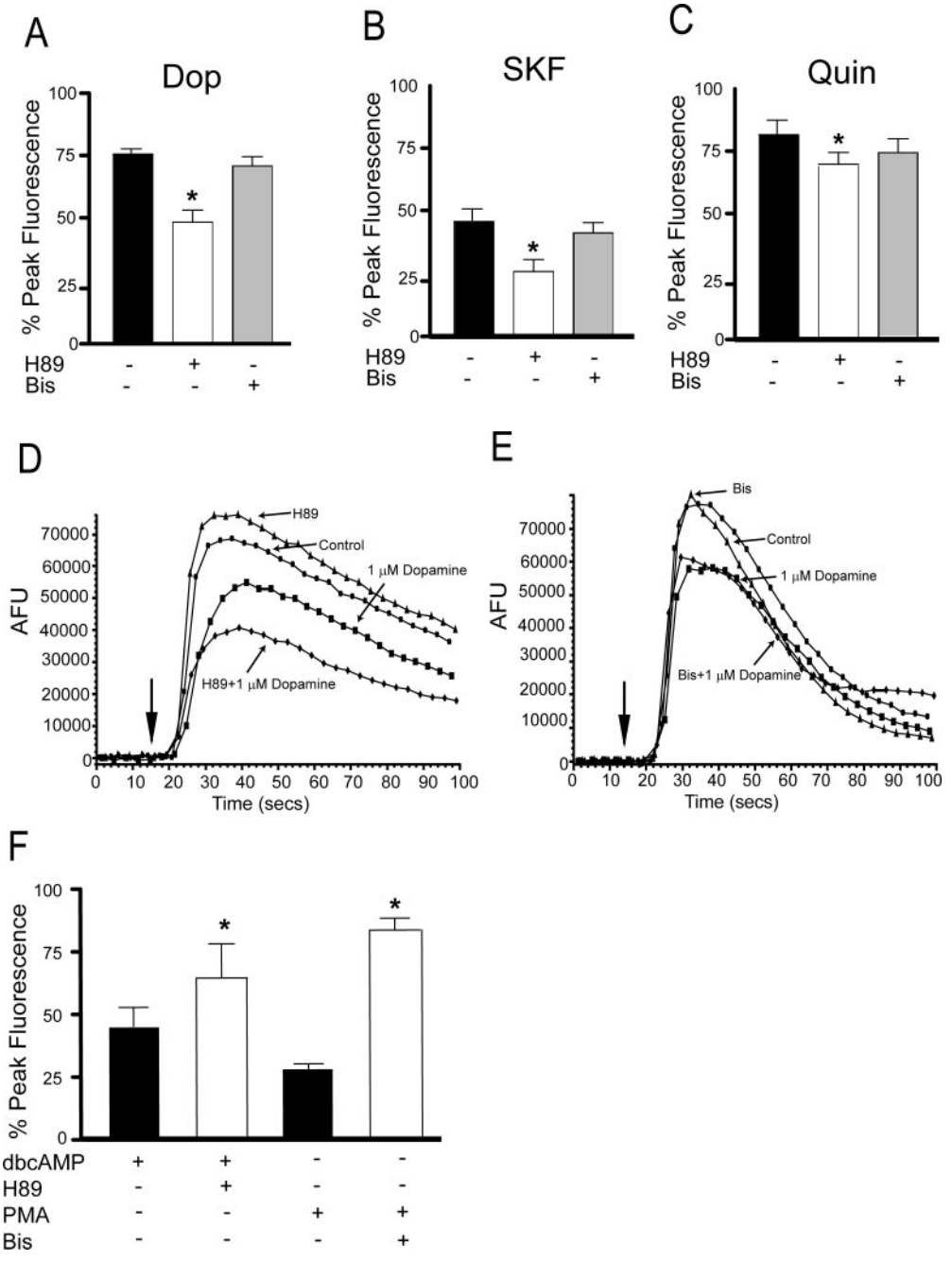

Fig. 5. Desensitization of the D1-D2–activated calcium signal was not decreased by inhibiting protein kinase A or protein kinase C. Desensitization of the calcium signal in cells pretreated with 1 μM dopamine (A), SKF81297 (B) quinpirole (C) without pretreatment of cells with inhibitors (black bar), with 1-h pretreatment with 10 μM H89 (white bar) or 500 nM Bis (gray bar) (n = 5–7). Representative calcium tracings for the effect of inhibiting PKA (D) and PKC (E) on dopamine-mediated desensitization of the calcium signal are shown. F, the calcium signal generated by 10 μM dopamine activation after the addition of 3 mM PKA activator dbcAMP or 1 μM PKC activator PMA (black bars). These effects are significantly decreased by cotreatment with 10 μM PKA inhibitor H89 or 500 nM PKC inhibitor Bis (white bars). Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle-pretreated cells ± S.E.M. (*, $p <$ 0.05).

was increased slightly but significantly by zinc pretreatment. To verify this result, the catalytic inert GRK2 mutant GRK2-K220R was expressed in D1-D2-expressing cells and was found to be unable to inhibit desensitization (data not shown).

Ca^{2+}-calmodulin kinase II (CAMKII) and casein kinases I and II, other second messenger-activated kinases that could potentially mediate desensitization, were tested for their involvement in the desensitization of the calcium signal. Cells were pretreated with 10 μM CAMKII inhibitor KN-93, 1 μM D4476 (a casein kinase I inhibitor), and 1 μM TBB (a casein kinase II inhibitor) before agonist pretreatment. The calcium signal associated with the activation of the D1-D2 hetero-oligomer with dopamine and activation of the P2Y1 receptor with ADPβS were both significantly attenuated by pretreatment with KN93, suggesting that this effect relates to the modulatory role CAMKII plays on the IP3 receptor, which had been shown previously (Aromolaran and Blatter, 2005) (D1R + D2R + dopamine = 79.6 ± 6.9 AFU × 10^3, D1R + D2R + dopamine + KN93 = 51.2 ± 5.3 AFU × 10^3, $n = 5$. $p < 0.05$. P2Y1R ± ADPβS = 87.2 ± 6.9 AFU × 10^3, P2Y1R±ADPβS + KN93 = 45.2 ± 6.5 AFU × 10^3, $n = 5$, $p < 0.05$). Treatment with KN-93 did not attenuate D1-D2 signal desensitization associated with dopamine, SKF81297, and quinpirole pretreatment (Fig. 9A). We were surprised to find that inhibiting CAMKII activity slightly but significantly increased the extent of desensitization in all cases. Because casein kinases are involved in desensitization of $G_{q/11}$ protein-linked receptors (Budd et al., 2000), cells were pretreated with 1 μM D4476, a casein kinase I inhibitor (Fig. 9B) or 1 μM TBB, a casein kinase II inhibitor

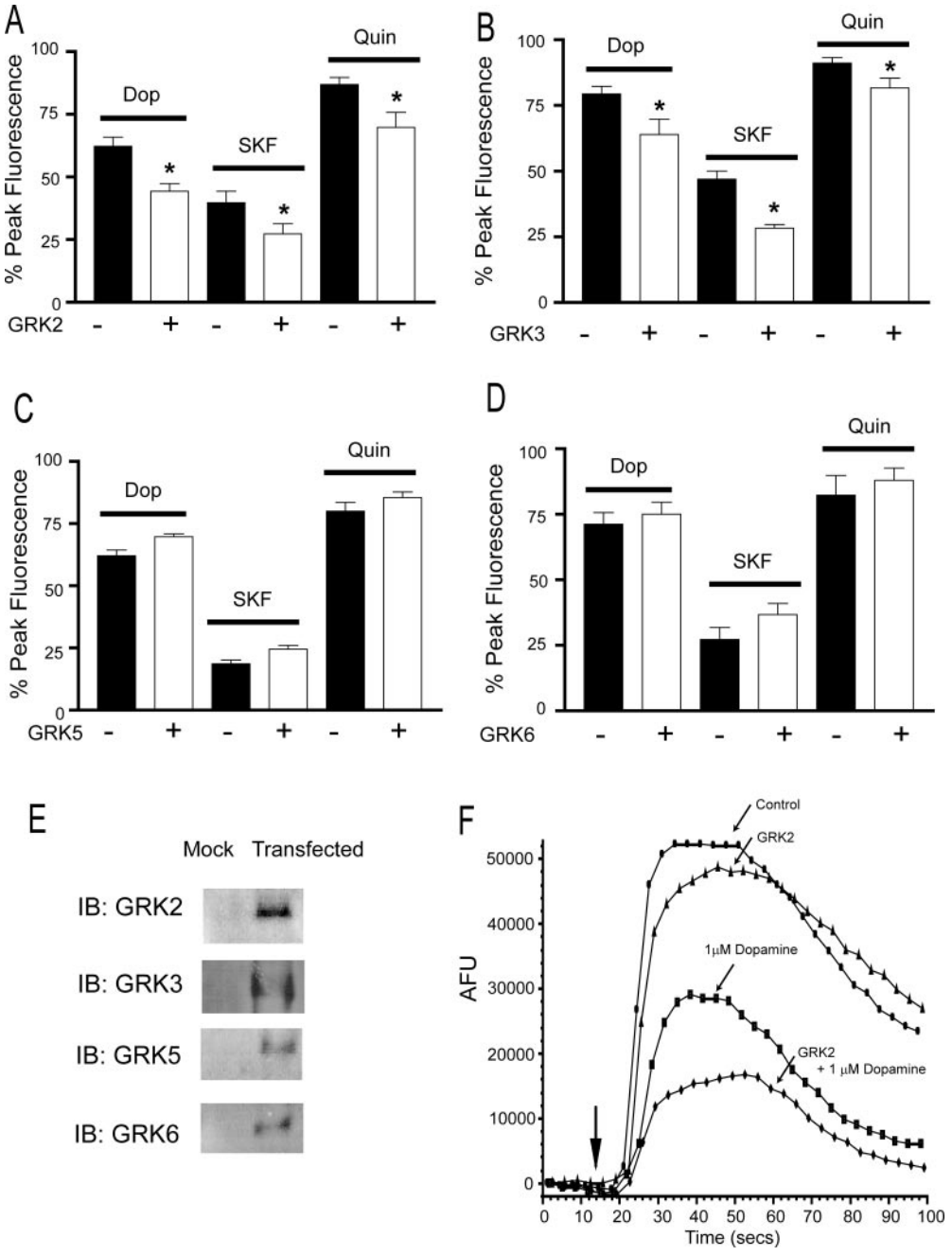

Fig. 6. Increased expression of G protein-coupled receptor kinases (GRK) 2 or 3, not 5 or 6, increased agonist-mediated desensitization of the D1-D2–activated calcium signal. Desensitization of the calcium signal after 30 min pretreatment with 1 μM dopamine, SKF81297, or quinpirole without (black bars) or with (white bars) increased expression of GRK2 (A), GRK3 (B), GRK5 (C), or GRK6 (D) ($n = 6$). E, immunoblot demonstrating increased expression of GRK2, GRK3, GRK5, or GRK6 in D1-D2–expressing cells transiently transfected with GRK constructs. Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle-pretreated cells ± S.E.M. (*, $p < 0.05$). F, representative calcium tracing for the effect of increased GRK2 expression on dopamine-mediated signal desensitization is shown.

(Fig. 9C). In both cases, the extent of desensitization was not decreased but was slightly but significantly increased.

Because the D1 and D2 receptors can couple to G_s and G_i proteins (Lee et al., 2004), the involvement of the activation of these proteins in the desensitization of the hetero-oligomer was investigated. The adenylyl cyclase inhibitor SQ22536 was used to inhibit G_s protein-mediated signaling activated by the D1 receptor and pertussis toxin (PTX) was used to selectively inhibit G_i protein-mediated signaling activated by the D2 receptor. Cells pretreated with 10 μM SQ22536 demonstrated no change in the extent of desensitization by any agonist tested (Fig. 10A). Treatment with 250 ng/ml PTX, however, selectively increased desensitization by dopamine and SKF81297 but not that mediated by quinpirole or ADPβS (Fig. 10B).

Discussion

Coactivation of D1 and D2 receptors within a D1-D2 hetero-oligomer generated a phospholipase C-linked calcium signal (Lee et al., 2004). In this report, we determined that the desensitization of this signal could be elicited by agonist occupancy of either receptor within the complex and occurred in under a minute or up to 10 min for the agonists used. This signal was homologously desensitized by pretreatment with dopamine, the D1-selective agonist SKF81297, or the D2-selective agonist quinpirole; the efficacy, rate, and potency of desensitization varied among the three agonists tested. Desensitization occurred independent of calcium storage capacity, exogenous calcium entry or receptor internalization, suggesting that modification of the hetero-oligomer itself allowed for signal desensitization. The inhibition of the second messenger- activated kinases PKA, PKC, CAMKII, or casein kinases I and II did not decrease signal desensitization. A role for GRK2 and -3, not GRK5 or -6, was established that was independent of their catalytic activity. G_i protein activity associated with the D2 receptor controlled the efficacy of the desensitization mediated by the D1 receptor within the D1-D2 complex.

Desensitization of the calcium signal occurred when either receptor within the D1-D2 complex was occupied by selective agonists. Characteristics of desensitization elicited were different, however, because SKF81297 treatment resulted in a more robust, more efficacious, and more rapid rate of desensitization compared with that after quinpirole treatment; dopamine displayed an intermediate response. This difference may be attributed to differential agonist induced conformations and the selectivity of these agonists for D1 and D2 receptors. Targeting either the D1 or D2 receptor within the D1-D2 complex resulted in desensitization characteristics reminiscent of the receptor when expressed alone. D1 homo-oligomers desensitized quickly, within minutes (Gardner et al., 2001; Lamey et al., 2002). D2 homo-oligomers, on the other hand, desensitized very slowly, requiring prolonged agonist treatment (Zhang et al., 1994; Ng et al., 1997). For the D1-D2 complex, when the D1 receptor was selectively targeted, the desensitization of the calcium signal occurred in less than 1 min; when the D2 receptor was selectively targeted, desensitization took approximately 10 min. It is noteworthy that the desensitization induced by dopamine, which targets both D1 and D2 receptors, displayed an intermediate

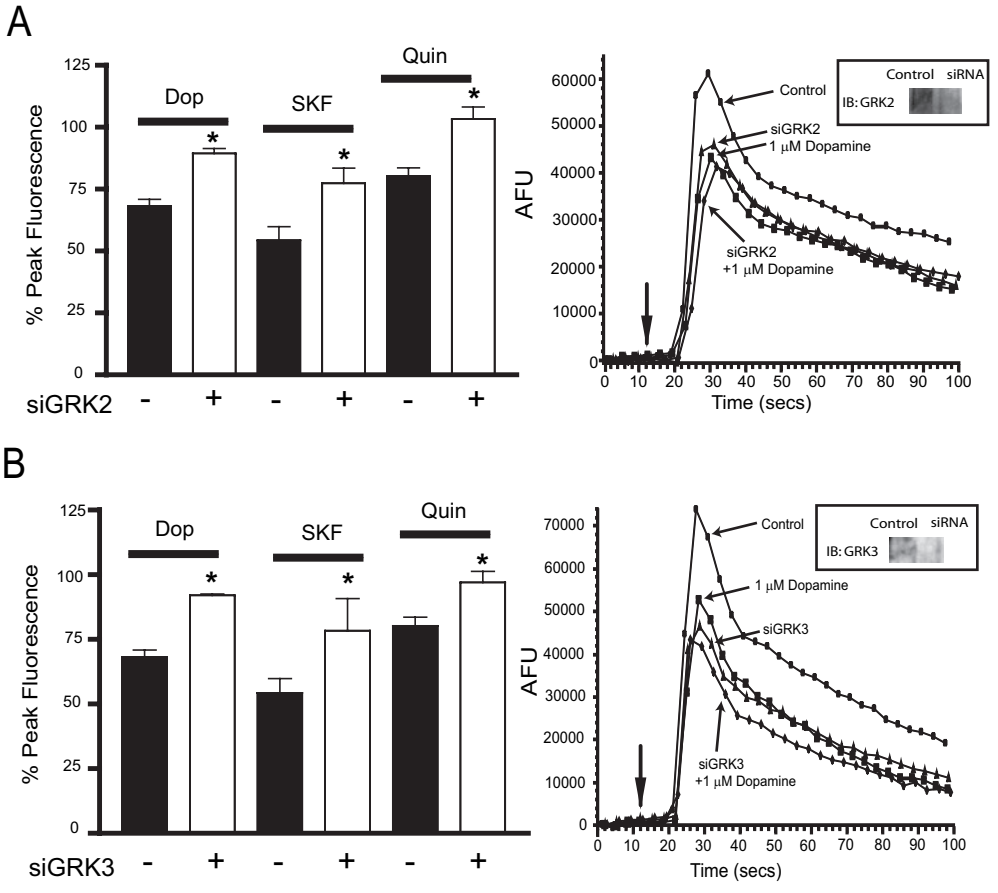

Fig. 7. Decreased expression of G protein-coupled receptor kinases 2 or 3 decreased agonist mediated desensitization of the D1-D2–activated calcium signal. Desensitization of the calcium signal after 30-min pretreatment with 1 μM dopamine, SKF81297, or quinpirole without (black bars) or with (white bars) decreased expression of GRK2 (A) or GRK3 (B) (n = 3–4). In each case, representative calcium tracings for dopamine-mediated signal desensitization are shown on the right panel. Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle-pretreated cells ± S.E.M. (*, $p < 0.05$). Insets, immunoblot demonstrating decreased expression of GRK2 and GRK3. Average silencing for these experiments was 65 to 70% of basal levels.

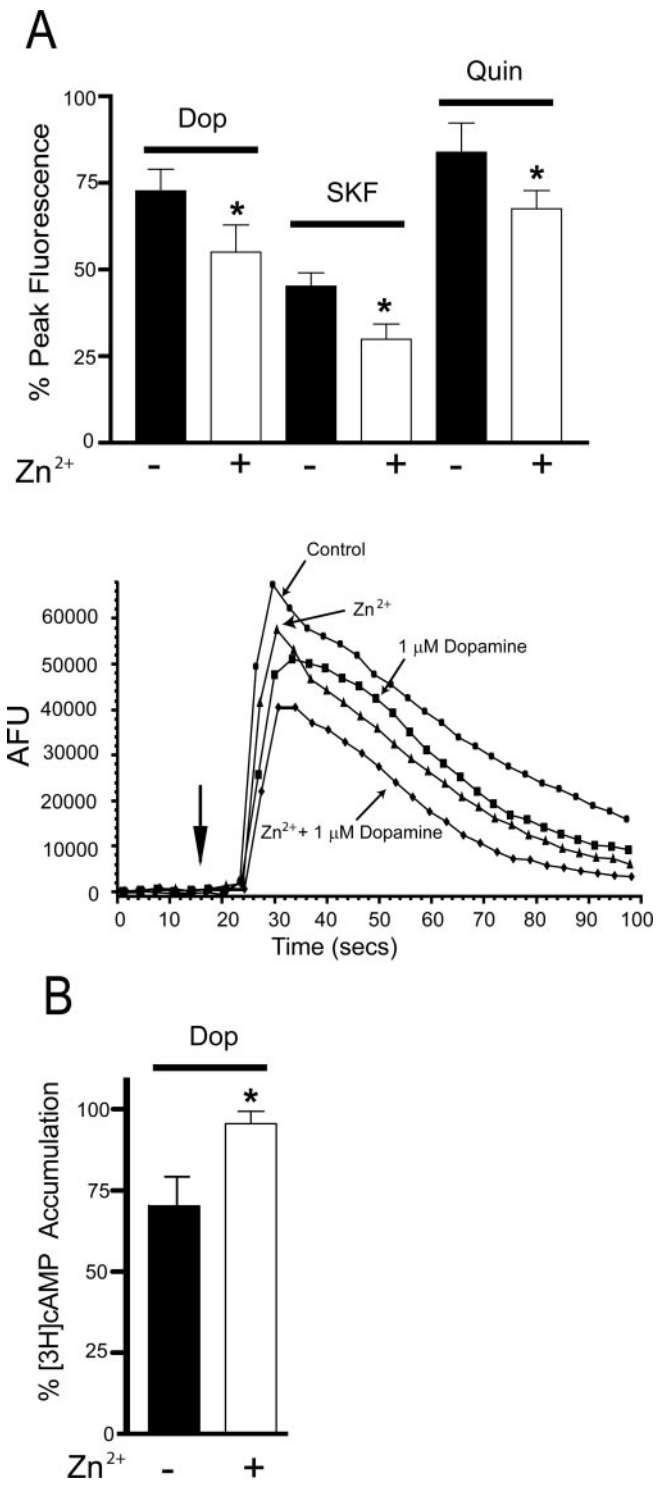

Fig. 8. The inhibition of the catalytic domain of G protein-coupled receptor kinases did not inhibit agonist-mediated desensitization of the D1-D2-activated calcium signal. A, the calcium signal activated by 10 μM dopamine after 30-min pretreatment with 1 μM dopamine, SKF81297, or quinpirole without (black bar) or with (white bar) 200 μM zinc chloride for 30 min ($n = 6$). Bottom, representative calcium tracing for the effect of zinc chloride on dopamine-mediated signal desensitization is shown. Data were represented as the percentage of peak fluorescence of the calcium signal from vehicle-treated cells ± S.E.M. B, [^{3}H]cAMP accumulation of the D1 receptor expressed alone pretreated with 1 μM dopamine for 30 min without (black bar) or with 200 μM zinc chloride (white bar). Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle-pretreated cells ± S.E.M. (*, $p < 0.05$).

profile, taking place in 5 min. This intermediate profile may be a distinct feature of dopamine-induced conformations or the result of its coactivation of D1 and D2 receptors. The activation of the D2 receptor within the hetero-oligomer may act as a braking mechanism to decrease the efficacy, rate, and efficiency of desensitization mediated by agonist occupancy of the D1 receptor.

This study also demonstrated that signal desensitization does not correlate with the efficacy of signal activation. Quinpirole desensitized the signal but did not independently activate calcium signaling. Desensitization mediated by dopamine or SKF81297 were both greater than that mediated by quinpirole, suggesting that the activation of the G_q-mediated signal may dictate the extent of desensitization. However, the observation that dopamine, which caused maximal activation of the signal, was not as efficacious in causing desensitization as SKF81297, which only partially activated the signal, suggests that there are factors beyond signal activation that mediate the efficacy of calcium signal desensitization. One potential factor may be the ability of each agonist to initiate protein kinase-mediated phosphorylation of the hetero-oligomer. For homo-oligomers, variable desensitization of their signals can stem from the ability of agonists to mediate differential phosphorylation of the receptor (Zhang et al., 1998). The lack of correlation between the ability of agonists to activate and their ability to desensitize receptor signaling has been reported for agonists within homo-oligomers (Barak et al., 2006) and this report is the first demonstration that this is also observed with GPCR hetero-oligomers.

Preventing agonist-mediated internalization of the complex did not decrease the extent of signal desensitization, which suggests that desensitization of the calcium signal has already occurred before recruitment of the complex into vesicles by endocytic machinery. We showed that a temporal dissociation of the processes of agonist-mediated desensitization and internalization existed for D1 receptor homo-oligomers (Ng et al., 1995). Instead of mediating desensitization, internalization may allow resensitization of receptors to occur through interactions with phosphatases in endosomes (Lefkowitz et al., 1998). This is a possibility, because attenuating the agonist-induced internalization of the hetero-oligomer increased signal desensitization, suggesting a loss of the resensitization process. Another possibility to consider is whether the D1-D2 receptor complex breaks apart during the desensitization process before receptor internalization, thus disrupting and turning off the signal.

The involvement of GRK2 and GRK3 in signal desensitization was confirmed by both increasing and decreasing the expression of these proteins in D1-D2–coexpressing cells. Inhibiting GRK-mediated phosphorylation of receptors, however, did not inhibit desensitization of the calcium signal. This suggests that, in addition to phosphorylating receptors, GRKs may also mediate signal desensitization by phosphorylation-independent mechanisms. For instance, GRK2 and GRK3 may sequester $G_{q/11}$ proteins, which interact with the RGS domain on these GRKs (Iwata et al., 2005), thereby eliciting signal desensitization by a phosphorylation-independent mechanism. GRKs can also desensitize the receptor without phosphorylation by sequestering $G\beta\gamma$ subunits and competing against phospholipase C β binding to activated forms of $G\alpha_q$ proteins (Tobin, 2002).

Because both D1 and D2 receptors contain motifs specific

for phosphorylation by a variety of kinases, their potential roles in the desensitization of the calcium signal was investigated. However, the ineffectiveness of their respective inhibitors to decrease homologous desensitization suggests that these kinases play no direct role in mediating homologous desensitization of the D1-D2 receptor complex. However, a potential modulatory role of some of these kinases is suggested by the ability of their respective inhibitors to increase (instead of decrease) calcium signal desensitization. We postulate that these kinases may alter the resensitization of the calcium signal. It has been demonstrated that blocking PKA and CAMKII phosphorylation of GPCRs inhibited receptor resensitization and increased desensitization (Hishinuma and Ogura, 2000; Tran et al., 2007). Despite the fact that inhibiting PKA and PKC activities did not affect homologous desensitization, these kinases are involved in heterologous desensitization of this signal. However, the activation of P2Y1 receptors was not able to heterologously desensitize this signal, suggesting that specific PKC isoforms that are not activated by P2Y1 receptors may be involved in this effect.

Desensitization is mediated by receptors coupled to a $G_{q/11}$ signaling pathway within the hetero-oligomeric complex and not by activation of G_s or G_i proteins. Inhibiting adenylyl cyclase activity did not decrease the desensitization mediated by dopamine or SKF81297, both of which activate adenylyl cyclases through the D1 receptor. Inhibiting the activation of G_i proteins by the D2 receptor did not decrease the desensitization mediated by quinpirole. However, inhibiting the ability of the D2 receptor to activate G_i proteins did result in increased desensitization mediated by agonists selectively targeting the D1 receptor. This suggests that downstream G_i protein signaling activated by D2 receptors may decrease desensitization resulting from D1 receptor activation. The

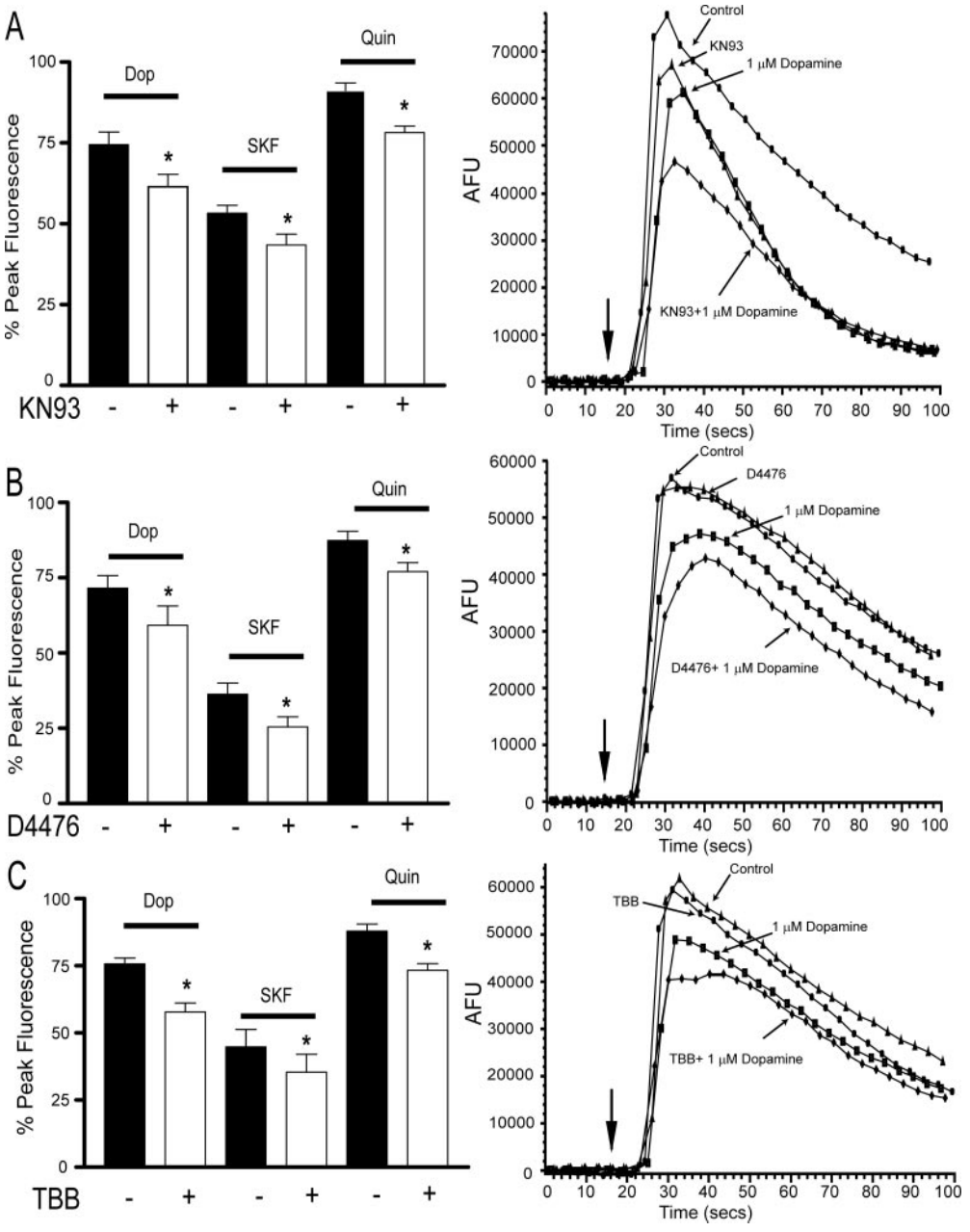

Fig. 9. The inhibition of CAMKII and casein kinases slightly increased agonist-mediated desensitization of the D1-D2–activated calcium signal. Desensitization of the calcium signal after 30-min pretreatment with 1 μM dopamine, SKF81297, or quinpirole without (black bars) or with (white bars) 10 μM KN93 (A), 1 μM D4476 (B), or 1 μM TBB (C) pretreatment for 1 h ($n = 5$). In each case, representative calcium tracings for dopamine-mediated signal desensitization are shown on the right panel. Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle-pretreated cells ± S.E.M. (*, $p < 0.05$).

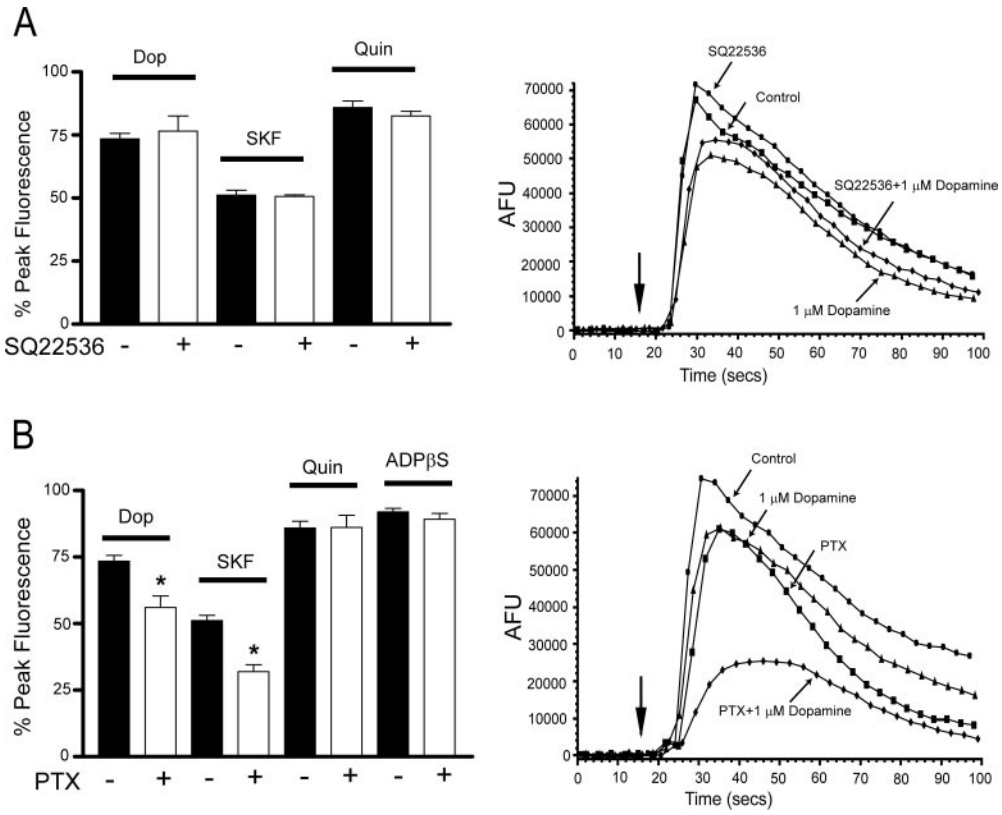

Fig. 10. Dopamine D1 receptor-mediated desensitization is increased by pertussis toxin pretreatment, but no effect on signal desensitization is observed with the adenylyl cyclase inhibitor SQ22536 (n = 4–5). A, desensitization of the calcium signal after 30-min pretreatment with 1 μM dopamine, SKF81297, or quinpirole without (black bars) or with (white bars) 10 μM SQ22536 (A) or overnight pretreatment with 250 ng/ml PTX (B). In each case, representative calcium tracings for dopamine-mediated signal desensitization are shown on the right panel. Data were represented as the percentage of peak fluorescence of the calcium signal generated by 10 μM dopamine in vehicle pretreated cells ± S.E.M. (*, $p < 0.05$).

activation of G_i proteins, and not G_s proteins, could accelerate the rate of resensitization of the $G_{q/11}$-coupled receptor and thus allow for decreased desensitization (Werry et al., 2003). It is not clear whether the G_i proteins activated are associated with D2 receptors within D2 homo-oligomers or within D1-D2 hetero-oligomers.

This study demonstrates for the first time the desensitization of a signal generated by a GPCR hetero-oligomer. These findings suggest different signaling regulation could result from selectively targeting either receptor within the complex, leading to differential development of tolerance and neuronal adaptation. Furthermore, our report showing differences in the effect of D1 or D2 receptor selective agonists on the calcium signal mediated by the D1-D2 receptor hetero-oligomer may have relevance for clinical conditions in which these selective agonists are used therapeutically. Continued investigation of this unique signal may yield a better understanding of behavioral and central nervous system disorders mediated by dopamine, such as drug addiction and schizophrenia. For instance, a large body of evidence suggests that dysfunction of calcium signaling may be associated with the etiology of schizophrenia (Lidow, 2003). Therefore, the elucidation of the control and termination of this signal may be integral to the understanding of normal physiology as well as disorders linked to dopamine-activated signaling systems.

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